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AF/1635

Attorney's Docket No. 032796-019

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
John P. Carulli *et al.*	Group Art Unit: 1635
Application No.: 09/578,900	Examiner: Jon E. Angell
Filed: May 26, 2000	Appeal No.:
For: HIGH BONE MASS GENE OF 11Q13.3	Confirmation No.: 8399

BRIEF FOR APPELLANT

Mail Stop APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This appeal is from the decision of the Primary Examiner dated November 23, 2003 (finally rejecting claims 1, 2, 6, 7 and 48-61, which are reproduced as Appendix A of this brief.)

A check covering the ☐ $165.00 (2402) ☒ $330.00 (1402) Government fee and two extra copies of this brief are being filed herewith.

The Commissioner is hereby authorized to charge any appropriate fees under 37 C.F.R. §§ 1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 02-4800. A copy of this page and the signature page are submitted in triplicate.

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Appendices

Appendix A: Appealed Claims

Exhibits

Exhibit A: Abstract of Zabaglia *et al.*, *Cad Saude Publica*, 14:779-86 (1998)

Exhibit B: Parhami *et al.*, *Journal of Bone Mineral Research*, 16:182-88 (2001).

Exhibit C: Fujino *et al.*, *Proceedings of the National Academy of Sciences USA*, 100:229-34 (2003)

Exhibit D: Magoori *et al.*, *J. Biol. Chem.*, Manuscript M211987200, published online on December 31, 2002

Exhibit E: Jin *et al.*, Trends in Endocrinology & Metabolism, 13:174-78 (2002)

Pursuant to 37 C.F.R. § 1.192, Appellants state the following:

I. REAL PARTIES IN INTEREST

The present application is assigned to Creighton University School of Medicine and Genome Therapeutics Corporation.

II. RELATED APPEALS AND INTERFERENCES

The Appellants' legal representative does not know of any other appeal or interferences which will affect or be directly affected by or have bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 1-61 are pending in the application. Claims 3-5 and 8-47 have been withdrawn from consideration as directed to non-elected subject matter. Claims 1, 2, 6, 7, and 48-61 are under examination and have been finally rejected.

IV. STATUS OF AMENDMENTS

No amendments have been filed subsequent to the final rejection. Claims in this application were amended by Appellants on August 25, 2003, February 23, 2003, August 12, 2002, and March 22, 2002. All amendments of record have been entered.

V. SUMMARY OF THE INVENTION

The Specification describes methods and materials used to isolate, detect and sequence a high bone mass gene (*HBM*) and corresponding wild-type gene (*Zmax1*), and variants thereof. The *HBM* gene is an allelic variant of the *Zmax1* gene. Persons having the *HBM* gene have been shown to exhibit a desirable lipid profile phenotype relative to persons having a corresponding wild-type *Zmax1* gene. *See*, for example, data presented in Example 3 of the Specification at pages 125-128. Thus, the inventors have discovered the high bone mass gene and have experimentally determined that the products of the *HBM* and *Zmax1* genes, (*i.e.*, the HBM protein, the Zmax1 protein, and variants thereof) are involved with modulating the levels of lipids in humans.

Accordingly, the invention generally relates to the *HBM* gene, the *Zmax1* gene, and their variants as well as the HBM and Zmax1 proteins and variants thereof. In view of the experimental data, and considering the features of the gene and its relationship with a family of receptors, the genes identified in the Specification are implicated in the ontology and physiology of atherosclerosis, arteriosclerosis and associated diseases and related conditions. The Specification also describes nucleic acids, proteins, cloning vectors, expression vectors, transformed hosts, methods of developing pharmaceutical compositions, methods of identifying molecules involved in arteriosclerosis and associated conditions, and methods of treating or preventing diseases associated with abnormal lipid levels.

In preferred embodiments, the presently appealed claims are directed to methods of identifying molecules involved in lipid regulation by identifying molecules that interact with HBM, Zmax1, or that interact with nucleic acids encoding HBM, Zmax1 including various variants thereof. Thus, the presently appealed claims utilize the discoveries described in the Specification in combination with techniques described in the Specification, and/or known in the art, to provide methods of identifying additional molecules that are involved in lipid regulation. Aspects of the methods defined by the appealed claims are described throughout the Specification as originally filed. For exemplary description of the claimed methods, the Board's attention is respectfully directed to pages 10-11, page 85, lines 3-9, pages 100-109, pages 115-117, and pages 125-128 of the Specification.

VI. THE ISSUE

The single remaining issue in the application is whether the methods of identifying a molecule involved in lipid regulation claimed in the present application have utility as required by 35 U.S.C. § 101, and correspondingly whether how to use the invention is enabled as required by 35 U.S.C. § 112, first paragraph. The final Office Action maintains a rejection of the claims under 35 U.S.C. § 101 as allegedly not supported by a substantial asserted utility or a well-established utility that was first set forth in an Office Action mailed April 23, 2003. *See*, Office Action Mailed November 26, 2003 at 3, § 7. The final Office Action also maintains a corresponding rejection under 35 U.S.C. § 112 that was first set forth in an Office Action mailed April 23, 2003, allegedly because one skilled in the art would not

know how to use an invention that is not supported by a substantial asserted utility or a well-established utility as alleged in the rejection under 35 U.S.C. § 101.

More particularly, the issue has been narrowed to whether or not the utility asserted in the Specification is credible in view of the data presented in the Specification, which is supported by, *inter alia*, a description of the relationship of the gene to genes of a family known to be associated with lipid metabolism. *See, Id.* at 4, § 11. The alleged basis of the rejection has been summarized by the Office in alleging that

> "The invention as claimed does not have a substantial asserted utility or a well establish[ed] utility because the specification fails to disclose the direct involvement of either HBM or Zmax1 in lipid regulation." *See*, Office Action Mailed April 23, 2003 at 7.

The Office has also stated that the rejection under 35 U.S.C. § 112, first paragraph, enablement turns on whether the biological involvement of HBM and Zmax1 in lipid regulation is established; stating, for example:

> "[S]hould the claimed use of the HBM and Zmax1 be found to be credible specific and substantial (or well-established), without a clear indication of the function of HBM and Zmax1 . . . one of skill in the art would still have to perform an undue amount of additionally experimentation in order to use HBM or Zmax1 as claimed. The amount of experimentation is deemed to be undue because involvement of HBM and Zmax1 in lipid regulation would have to be established before one could attempt to practice the claimed invention." *See, Id.*

While it is recognized that enablement and utility are separate requirements, the only reasons that have been alleged for the rejections under appeal concern the credibility of the asserted utility. [1]

VII. GROUPING OF CLAIMS

Claims 1, 6, and 53 are independent claims; claims 2, 7, 48-52 and 54-61 are dependant claims. With respect to the issue of utility under appeal, Claims 1, 2, 6, 7, and 48-61 stand or fall together.

---

[1] It is worth noting that the present rejection, presented in the second Office Action on the merits, mailed April 23, 2003, reflects a position that is the polar opposite of the Office's position in the first Action on the merits mailed September 3, 2002. While the Office originally asserted that the utility of the presently claimed methods would have been so credible, based on sequence homology alone, as to be allegedly obvious to one of skill in the art, the Office now takes the position that even the biological data disclosed in the Specification does not credibly establish that HBM and Zmax1 are involved in lipid regulation.

VIII. ARGUMENT

A. INTRODUCTION

The final rejection of Claims 1, 2, 6, 7, and 48-61 under 35 U.S.C. §§ 101 and 112, first paragraph, is hereby appealed. With regard to 35 U.S.C. § 101, the Office first alleged in the Action mailed April 23, 2003, that the appealed claims are not supported by either a substantial asserted utility or a well-established utility. *See,* Office Action mailed April 23, 2003 at 4. With regard to 35 U.S.C. § 112, first paragraph, it was further alleged that if the claims are not supported by either a substantial asserted or a well-established utility, one skilled in the art would not know how to use the claimed invention. *See, Id.*

Appellants respectfully submit that the rejections should be reversed because the Office has failed to meet the initial burden of challenging a presumptively correct assertion of utility in the disclosure. *In re Brana*, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995)(citing, *In re Marzocchi,* 169 U.S.P.Q. 367, 369-370 (C.C.P.A. 1971)). A patent examiner must accept a utility asserted by an applicant unless the examiner has evidence or sound scientific reasoning to rebut the assertion. *See, In re Oetiker*, 1445, 24 U.S.P.Q.2d 1443, 1444 (Fed. Cir. 1992). Only after the Office provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. *In re Brana* 34 U.S.P.Q.2d at 1441 (citing, *In re Bundy,* 433, 209 U.S.P.Q. 48, 51 (C.C.P.A. 1981)).

Moreover, in failing to give proper credit to the direct biological evidence that the claimed methods have a credible, specific and substantial utility, which is presented in the Specification, and which is supported by additional circumstantial evidence described in the Specification and independent biological evidence, the Examiner has misapplied the standard of compliance with 35 U.S.C. § 101 which has been set forth by the courts and interpreted by the United States Patent Office in the *Utility Examination Guidelines*. *See, e.g., In re Brana, supra; Utility Examination Guidelines*, 66 F.R. 1092 (2001); and *Juicy Whip Inc. v. Orange Bang Inc.*, 51 U.S.P.Q.2d 1700 (Fed. Cir. 1999).

B. THE ASSERTED UTILITY IS A SUBSTANTIAL AND SPECIFIC UTILITY

The Claims under appeal are directed to methods of identifying molecules involved in lipid regulation. That the identification of molecules involved in lipid regulation is a specific and substantial utility is not disputed by the Office. The *Utility Examination Guidelines* explains that the requirement of a specific and substantial utility excludes "throw-away," "insubstantial," or "nonspecific" utilities such as the use of a complex invention as landfill. *Utility Examination Guidelines*, 66 F.R. 1092, 1098 (2001).

The methods of the presently appealed Claims are clearly not "throw-away" methods. The methods are specific, because the asserted utility is directly and specifically related to the function of HBM and Zmax1 in mediating lipid regulation that is described in the Specification. The methods claimed in the appealed Claims are substantial, because the Claims are directed to identifying molecules that themselves have specific and substantial utility as molecules that are involved in lipid regulation. The Office has not alleged that the identification of a molecule involved in lipid regulation is not a specific and substantial utility.

C. THE ASSERTED UTILITY IS CREDIBLE

The alleged basis of the rejection has been summarized by the Office as "[T]he specification fails to disclose the direct involvement of either HBM or Zmax1 in lipid regulation." *See,* Office Action mailed April 23, 2004 at 7. However, contrary to the allegations in support of the rejections, the involvement of HBM and Zmax1 in lipid regulation is clearly disclosed in the application. *See, e.g.,* Specification, at 10-11 and 85, lines 3-9. Furthermore, the credibility of the asserted utility is supported by experimental data disclosed in the specification. *See, e.g.*, Specification at 125-128.

It is worth briefly stating the following background. The Zmax1 and HBM proteins are related; HBM is a polymorphic variant of Zmax1. HBM has a glycine to valine change at residue 171 of Zmax1. *See,* Specification at 18-19. The polymorphism was found in a family identified as having a significantly elevated bone mass. *See,* Specification at 123-4. The discovery of the kindred and identification of the genetic basis of the high bone mass trait is described in the Specification in great detail. *See*, Specification at 28-74. The HBM

phenotype (*i.e.*, trait) was defined by those individuals having a statistically significant increase in bone mass density. *Id.* Through extensive experimentation, it was discovered that members of the kindred that have the HBM variant of Zmax1 display the high bone mass phenotype. *See*, Specification at 73-74. Thus, it has been established that a single altered amino acid of Zmax1 is sufficient to confer a phenotypic change in bone mass density. Thereby, it has been further established by the Appellants that Zmax1 is involved in the regulation of bone mass density and that the HBM polymorphism of Zmax1 is responsible for the HBM phenotype.

Zmax1 and the HBM variant are identified as transmembrane receptors related to the low density lipoprotein (LDL) receptor. *See*, Specification, at Figure 4. The Specification teaches that this relationship is consistent with a signaling protein function for Zmax1. *See*, Specification at 83. Following a discussion of several properties of Zmax1, the specification asserts that "it is likely that molecules that bind to Zmax1 may usefully alter bone development and lipid levels. Such molecules may include, for example, small molecules, proteins, RNA aptamers, peptide aptamers, and the like." *See,* Specification at 85.

1. The credibility of the asserted utility is supported by experimental data.

Experiments were conducted on samples obtained from members of the HBM kindred to determine whether Zmax1 and HBM were involved in lipid regulation. These experiments are disclosed and analyzed in Example 3 of the Specification at pages 125 to 128. Standard diagnostic protocols were used. The data is presented in the Table at page 128 of the Specification.

The data disclosed in the Specification clearly establish that the HBM polymorphism of Zmax1 is associated with an altered lipid profile in addition to its role in bone mass modulation. At least the following was determined: That affected members (*i.e.*, members having the HBM polymorphism of Zmax1) had statistically significant reductions in triglyceride levels, and reduced very low density lipoprotein (VLDL) levels. *See*, Specification at 127. Further, at a high level of confidence ($p=0.06$; 94% confidence), it was also found that males having the HBM polymorphism had increased high density lipoprotein levels (HDL) levels. The ratio of LDL to HDL is significantly different in those with the

HBM variant of Zmax1 than normal persons with Zmax1. *Id.* Therefore, the role of Zmax1 and HBM in lipid regulation is very credibly established.

2. *The credibility of the asserted utility is consistent with knowledge in the art.*

The data disclosed in the specification is consistent with the knowledge in the art at the time the application was filed. For example, the Zmax1 protein has a degree of sequence homology and features in common with the LDL receptor. *See*, Specification at pages 83-84. This sequence homology has been acknowledged by the examiner as consistent with the asserted utility. In the first Office Action on the merits, the Office set forth a rejection under 35 U.S.C. § 103, alleging that the methods of claims 1 and 2 would have been obvious over Dong *et al.*, *Biochem. and Biophys. Research Communications*, 251:784-90 (1988) in view of U.S. Patent No. 5,283,173 (1994) to Fields *et al.* Dong *et al.* described a gene and protein designated LR3 that has a substantial amount of identity to Zmax1. LR3 was described as a member of the low density lipoprotein (LDL) receptor family by Dong *et al.* Fields *et al.* discloses a method of identifying proteins that bind to a target protein. In the first Action on the merits, the Office alleged that membership in the LDL receptor family alone was sufficient to render the claims obvious. Appellant's Reply successfully pointed out that Dong *et al.* did not teach a function for LR3 and that there were enough differences in structure and function in the LDL receptor family that the familial association, standing alone, could not establish a *prima facie* case of obviousness. Thus, the prior art was insufficient to support a rejection under 35 U.S.C. § 103. However, when combined with observations and insights disclosed in the Specification, for example the experimental data disclosed at pages 125-128, the relationship to the LDL receptor does add support to the credibility of the conclusions indicated by the experimental results.

The data disclosed in the Specification is also consistent with observed protein binding interactions of Zmax1. The Specification discloses that Zmax1 binds to several proteins including apolipoprotein E (ApoE). *See,* Specification at 115. As its name suggests, ApoE is a protein associated with lipids in lipoprotein complexes. The observation of ApoE binding to Zmax1 provides further evidence of the credibility of the asserted utility.

Further circumstantial evidence adds to the credibility of the asserted utility. For example, the English language abstract of Zabaglia *et al.*, *Cad Saude Publica*, 14:779-85, 1998 (attached as Exhibit A), shows correlations between certain lipid profile parameters and bone mineral density. Zabaglia *et al.* reports that in postmenopausal women, HDL levels showed an inverse correlation to bone mass to a very high degree of statistical significance ($p=0.001$). High total cholesterol had a positive association with bone mineral density ($p=0.026$), and the LDL:HDL ratio showed a negative association with bone mineral density ($p=0.002$). The appearance of correlations between bone mineral density and lipid profile parameters is consistent with a single pathway affecting both. The results of Zabaglia *et al.* are consistent with a lipid profile regulatory mechanism and a bone mass regulation pathway sharing a common mediator, such that a change in that mediator can affect both lipid regulation and bone mass.

The conclusion by Zabaglia *et al.* that lipid profile parameters are generally not useful as a diagnostic indicator of bone mass does not contradict the data disclosed in the present application. Bone mass varies within the group having the HBM gene, as does lipid parameters. However, the mean parameters demonstrate a correlation. As can be seen from the table on page 128 of the Specification, the Z-score of bone mass varies within the unaffected group (*i.e.*, normal persons). Likewise, the lipid profile parameters varied within the unaffected group. Due to the variance within the groups, a lipid profile parameter may not be a precise predictor of bone mass. Nevertheless, when mean parameter values of the affected and unaffected groups are compared, the correlation between bone density and lipid profile becomes clear. The correlations between Z-score and lipid profile parameters provides significant support for Appellants' discovery of the involvement of Zmax1 and HBM in lipid regulation in addition to bone regulation.

3. Independent reports confirm the asserted utility.

The credibility of the asserted utility has been borne out in reports in the literature. For example, Parhami *et al.*, *J. Bone & Min. Res.*, 1:182-8, 2001 (Attached as Exhibit B) surveyed the history of links between lipid regulation and bone mineral density in their introductory section. Parhami *et al.* cites publications from long before the filing date of the present application (i.e. 1972 and 1992) as demonstrating that osteoperosis and

cardiovascular disease are linked regardless of age. *See,* Parhami *et al.* at first sentence. Publications from 1991, 1993 and 1999 are cited to show that low bone mineral density is associated with cardiovascular disease mortality. The credibility of the asserted utility is further confirmed by recent studies. For example, Fujino *et al.*, *PNAS USA*, 100:229-234, 2003 (Attached as Exhibit C) and Magoori *et al.*, *J. Biol. Chem.*, Manuscript M211987200, published online on December 31, 2002 (Attached as Exhibit D), have shown that Zmax1 (now called LRP5) is essential for normal cholesterol metabolism.

4. The Office has not met the initial burden to show that one of skill in the art would have a reason to doubt the asserted utility.

The Office has not provided a reason and evidence to show that one of skill in the art would have a reason to doubt the utility asserted in the Specification as required. *See, In re Oetiker*, 1445, 24 U.S.P.Q.2d 1443, 1444 (Fed. Cir. 1992). Despite the data presented in the Specification, the Office alleged in the Action mailed April 23, 2003 that "[t]he only link between HBM and lipid regulation is the indication that persons with the HBM polymorphism show a generally lower serum level of triglycerides and VLDL and a generally higher serum level of HDL, compared to controls." Office Action mailed April 23, 2003 at 5. Appellants submit that the disclosure of such *in vivo* data is more than sufficient to credibly establish the involvement of HBM and Zmax1 in lipid regulation.

Further, despite the clear relationship between Zmax1 and HBM (being variants of the same gene and differing at a single critical site), the Examiner alleges the "the only link between Zmax1 and lipid regulation is sequence homology between Zmax1 and the LDL receptor." *Id.*. However, the experimental data is equally relevant to the role of wild-type Zmax1, as well as the HBM polymorphism in lipid regulation. Appellants respectfully submit that since the difference in observed lipid profiles is seen to correspond to the difference between the presence or absence of the HBM polymorphism in Zmax1, the link between Zmax1 and HBM and lipid regulation has thereby been equally well-established.

5. The references cited as allegedly supporting the rejections do not provide a reason to doubt the asserted utility.

The references cited in the Office Action mailed April 23, 2003 in support of the appealed rejections do not meet the burden of showing that one of skill in the art would have a reason to doubt the credibility of the asserted utility. For example, Ye *et al.*, *Am. J. Clin.*

Nutr., 72:1275S-1284S, 2000 teaches that genes influence quantitative variations in plasma lipoprotein concentrations. Ye *et al.* reviews a series of polymorphisms in various genes involved in lipid regulation. In the section cited on page 6 of the Office Action mailed April 23, 2003, Ye *et al.* reports that studies of the effects of dietary cholesterol have not been consistent due to a series of confounding factors. This only means that it remains to be determined under what circumstances and for which polymorphisms a dietary intervention is indicated.

However, Ye *et al.* does not in any way cast doubt on whether those polymorphisms, or the HBM polymorphism, appear in genes related to lipid regulation. Ye *et al.* only shows that diet alone may not have a consistent effect. It certainly does not provide a reason to doubt the utility of the present invention. The question of whether a dietary change can affect lipid profiles simply has no bearing on the question of whether identifying a molecule that binds to a protein involved in lipid regulation (such as HBM or Zmax1) has credible utility as a method for identifying a molecule that is involved in lipid regulation.

Willnow *et al.*, *Nature Cell Biol.*, 1:E157-E162, 1999 is also cited in support of this rejection on page 6 of the Office Action mailed April 23, 2003. Willnow *et al.* teaches that members of the LDL receptor family of proteins have many varied functions. However, as has been stated, the assertion of utility in the present Application is supported by, but does not rest on, familial association. The credibility of the asserted utility is supported by biological *in vivo* data, which is not contested by this publication. This publication may demonstrate that the claimed methods are not obvious, but provides no reason to doubt the credibility of the asserted utility of the claimed invention.

6. *Identification of molecules that bind to a protein involved in lipid regulation is a well-established utility.*

The utility of screening for other molecules that bind to or inhibit binding to a protein is a well-established utility once the involvement of that protein in regulation of an important metabolic parameter has been identified. Such methods provide useful identification of potential therapeutic and/or therapeutic target molecules. Jin *et al.*, *Trends Endrocrin. & Metab.*, 13:174-8, 2002 (attached as Exhibit E) exemplifies the value that those skilled in the art place on discovering new molecules involved in lipid regulation. Jin *et al.*, reviews the

state of the art in the identification of lipases that affect levels of high density lipoproteins (HDL). HDL are implicated in protection from cardiac disease. For example, Jin *et al.* cites publications from 1994-1999 reporting that a single nucleotide polymorphism in hepatic lipase (HL) has been associated with increased levels of high density lipoprotein cholesterol (HDL-C). See, Jin *et al.* at 175, second column. Jin *et al.* describes the value of the discovery of these lipases as follows:

> "Because of their intimate relationship with HDL metabolism and function, [these lipases] are likely to have important effects on atherosclerosis in humans. Indeed, several of these lipases are viable targets for new drug development." Id. at 177.

As described in the Specification, for example at pages 115-117, the identification of molecules, such as proteins, that bind to HBM and Zmax1 can be used to identify molecules that are involved in lipid regulation. Such a utility would be recognized by one skilled in the art as a well-established specific and substantial utility.

7. The requirements of both 35 U.S.C. §§ 101 and 112, first paragraph have been met.

With regard to the rejection under 35 U.S.C. § 112, first paragraph, the Office has alleged that "without a clear indication of the function of HBM and Zmax1, particularly with respect to lipid regulation, one of skill in the art would still have to perform an undue amount of additional[] experimentation in order to use HBM or Zmax1 as claimed." However, the Office has not provided an analysis of the factors that is required to support such an assertion. *See, e.g. In re Wands*, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988). Rather, the only reason that is alleged for holding that additional experimentation would be undue is that "involvement of HBM and Zmax1 in lipid regulation would have to be established before one could attempt to practice the claimed invention."

Appellants respectfully submit that, as demonstrated above, the involvement of HBM and Zmax1 in lipid regulation has been credibly established by the data disclosed in the Specification considered in view of additional observations disclosed in the Specification and the knowledge in the art at the time the application was filed. Furthermore, the involvement of HBM and Zmax1 in lipid regulation has been confirmed by separate independent studies.

A credible specific and substantial utility is asserted in the Specification for the presently claimed methods. Moreover, the utility of the presently claimed methods would be readily apparent to one of skill in the art as a well-established utility in view of the data and observations disclosed in the specification. The Office has failed to meet the burden of making a *prima facie* showing that one skilled in the art would have any reasonable basis to doubt the asserted utility. However, even if the Office did meet the burden of establishing a *prima facie* showing of lack of credibility, the data disclosed in the Specification taken together with the supporting evidence would be sufficient to rebut the assertion. Thus, the requirements of 35 U.S.C. § 101 and the corresponding requirements of 35 U.S.C. § 112, first paragraph have been met. *See, e.g. Utility Examination Guidelines*, 66 F.R. 1092 (2001)

IX. CONCLUSION

For at least the reasons set forth above, the rejections of claims 1, 2, 6, 7, and 48-61 under 35 U.S.C. §§ 101 and 112, first paragraph, are improper and should be reversed. As this is the single remaining issue in the present application, an order reversing the rejections and directing that a notice of allowance should be issued with appropriate dispatch is respectfully requested.

Respectfully submitted,

Burns, Doane, Swecker & Mathis, L.L.P.

Date July 2, 2004

By: _____
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APPENDIX A

The Appealed Claims

Claim 1. A method of identifying a molecule involved in lipid regulation comprising identifying a molecule that binds to, or that inhibits binding of a molecule to, HBM or Zmax1.

Claim 2. The method of claim 1, wherein said molecule is a protein.

Claim 6. A method for identification of a candidate molecule involved in lipid regulation comprising:

(A) identifying a first molecule that binds to, or that inhibits binding of a second molecule to, the nucleic acid sequence of either (i) a Zmax1 nucleic acid chosen from among the sequence SEQ ID NO: 1 and a Zmax1 nucleic acid comprising a polymorphism of Table 4, except for the C/A base change at location 21119 (308G), or (ii) a HBM nucleic acid having SEQ ID NO: 2;

(B) measuring the binding of the first molecule, or inhibition of the binding of the second molecule, to the other of either (i) the Zmax1 nucleic acid or (ii) the HBM nucleic acid; and,

(C) comparing the extent of binding of the first molecule, or the extent of inhibition of binding the second molecule, to each nucleic acid sequence, wherein the molecule that binds, or inhibits binding, more or less to the HBM nucleic acid sequence of SEQ ID NO: 2 versus the Zmax1 nucleic acid sequence of SEQ ID NO: 1 or a Zmax1 nucleic acid comprising a polymorphism of Table 4, except for the C/A base change at location 21119 (308G), is the candidate molecule.

Claim 7. The method of claim 6, wherein the candidate molecule is a protein, an mRNA or an antisense nucleic acid.

Claim 48. The method of claim 1, wherein the HBM or Zmax1 is in solution.

Claim 49. The method of claim 1, wherein the HBM or Zmax1 is affixed to a solid support.

Claim 50. The method of claim 1, wherein the HBM or Zmax1 is located on a cell surface.

Claim 51. The method of claim 1, wherein the HBM or Zmax1 is expressed by a host cell.

Claim 52. The method of claim 48, wherein the molecule which binds to HBM or Zmax1 is identified by assaying the competitive binding of the molecule to HBM or Zmax1 in the presence of a known ligand.

Claim 53. A method of identifying a molecule involved in lipid regulation comprising identifying a molecule that binds to, or that inhibits binding of a molecule to, HBM (SEQ ID NO:4).

Claim 54. The method of claim 2, wherein the molecule is identified by co-immunoprecipitation with HBM or Zmax1.

Claim 55. The method of claim 2, wherein the molecule is identified by co-fractionation with HBM or Zmax1.

Claim 56. The method of claim 2, wherein the molecule is identified by a two-hybrid system in which the extracellular domain of HBM or Zmax1 is encoded on a bait vector.

Claim 57. The method of claim 1, wherein the molecule binds to, or inhibits binding of a molecule to, HBM more or less than to Zmax1.

Claim 58. The method of claim 1, wherein the step of identifying a molecule that binds to, or that inhibits binding of a molecule to, HBM or Zmax1 is a step of identifying a molecule that binds to, or that inhibits binding of a molecule to HBM.

Claim 59. The method of claim 1, wherein the step of identifying a molecule that binds to, or that inhibits binding of a molecule to, HBM or Zmax1 is a step of identifying a molecule that binds to, or that inhibits binding of a molecule to Zmax1.

Claim 60. The method of claim 1 wherein the step of identifying a molecule that binds to, or that inhibits binding of a molecule to, HBM or Zmax1 is a step of identifying a molecule that binds to, or that inhibits binding of a molecule to HBM and to Zmax1.

Claim 61. The method of claim 1 further comprising the step of determining whether the molecule that binds to, or that inhibits binding of a molecule to, HBM or Zmax1 is a molecule that binds to, or that inhibits binding of a molecule to HBM to a greater or lesser extent than to Zmax1, and wherein the molecule involved in lipid regulation is a molecule that binds to, or that inhibits binding of a molecule to HBM to a greater or lesser extent than to Zmax1.

1: Cad Saude Publica. 1998 Oct-Dec;14(4):779-86.

[An exploratory study of the association between lipid profile and bone mineral density in menopausal women in a Campinas reference hospital]

[Article in Portuguese]

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A total of 72 postmenopausal patients presenting no risk factors for cardiovascular disease nor osteoporosis, were studied. The study evaluated total serum cholesterol and fractions and bone mass by densitometry of the lumbar spine and femur using a Lunar-DPX. There was no association between lipid profile variables and bone mineral density, except for high density lipoprotein (HDL), which showed an inverse correlation (p=0.001). Multiple regression showed that total cholesterol levels higher than 240 mg% had a positive association with BMD (p=0.026). In addition, the ratio between LDL and HDL (Castelli 2 index) showed a negative association with BMD (p=0.002). The diagnostic validation test showed that all lipid profile variables had low sensitivity and specificity as indicators for osteoporosis. The conclusions were that lipid profile variables did not show a significant association with bone mass and could not be used as indicators for bone mineral density.

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Atherogenic High-Fat Diet Reduces Bone Mineralization in Mice

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ABSTRACT

The epidemiological correlation between osteoporosis and cardiovascular disease is independent of age, but the basis for this correlation is unknown. We previously found that atherogenic oxidized lipids inhibit osteoblastic differentiation in vitro and ex vivo, suggesting that an atherogenic diet may contribute to both diseases. In this study, effects of an atherogenic high-fat diet versus control chow diet on bone were tested in two strains of mice with genetically different susceptibility to atherosclerosis and lipid oxidation. After 4 months and 7 months on the diets, mineral content and density were measured in excised femurs and lumbar vertebrae using peripheral quantitative computed tomographic (pQCT) scanning. In addition, expression of osteocalcin in marrow isolated from the mice after 4 months on the diets was examined. After 7 months, femoral mineral content in C57BL/6 atherosclerosis-susceptible mice on the high-fat diet was 43% lower (0.73 ± 0.09 mg vs. 1.28 ± 0.42 mg; $p = 0.008$), and mineral density was 15% lower compared with mice on the chow diet. Smaller deficits were observed after 4 months. Vertebral mineral content also was lower in the fat-fed C57BL/6 mice. These changes in the atherosclerosis-resistant, C3H/HeJ mice were smaller and mostly not significant. Osteocalcin expression was reduced in the marrow of high fat-fed C57BL/6 mice. These findings suggest that an atherogenic diet inhibits bone formation by blocking differentiation of osteoblast progenitor cells. (J Bone Miner Res 2001;16:182–188)

Key words: **osteoporosis, oxidized lipids, bone, atherosclerosis, high-fat diet**

INTRODUCTION

EPIDEMIOLOGICAL EVIDENCE links osteoporosis with cardiovascular disease, independently of age.[1,2] Osteoporosis and the subsequent 1 million fractures in the United States each year[3] results from a combination of increased bone resorption and decreased bone formation. Low bone mineral density (BMD) is associated closely with cardiovascular disease mortality,[4–6] cardiovascular calcification,[7–9] atherosclerosis,[10,11] and high lipid levels.[10–13] Such correlations raise the possibility of a common underlying factor or mechanism.

We previously found that minimally oxidized low-density lipoprotein (MM-LDL), and other bioactive oxidized lipids that promote atherogenesis and are increased in atherosclerotic lesions,[14–19] also inhibit osteoblastic differentiation of bone- and marrow-derived preosteoblasts in vitro.[20,21] Preosteoblasts harvested from the marrow of mice fed a high-fat, atherogenic diet showed significantly less osteoblastic differentiation.[21] Others have shown a paucity of cells committed to the bone lineage in osteoporotic bone marrow and with aging.[22,23] These links between lipids, vascular disease, and bone suggest the novel hypothesis that oxidized lipids are the biological link.

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In this study, effects of a high-fat atherogenic diet versus control chow diet on bone mineral content (BMC) and BMD were tested in two strains of mice with genetically different susceptibility to oxidized lipids and atherogenesis. In 1985, Paigen et al. showed differences in the susceptibility of two inbred strains of mice to development of hyperlipidemia and atherosclerotic lesions when fed an atherogenic diet[24,25]; C3H/HeJ were identified as a resistant strain and C57BL/6 as a sensitive strain. Several years later, Liao et al. reported the induction of inflammatory genes by an atherogenic diet in the C57BL/6 but not in the C3H/HeJ strain,[26] and Navab et al.[27] and Shih et al.[28] found differences in the antioxidant defense systems between the susceptible and resistant mouse strains. In the present study, we have compared the susceptibility and resistance of these two strains of mice to the effects of high-fat diet-induced hyperlipidemia on bone. We report that in the atherosclerosis-susceptible C57BL/6 mice, BMC and BMD were significantly lowered by the high-fat diet versus chow diet. These changes were smaller in the atherosclerosis-resistant C3H/HeJ mice. In addition, marrow cells from the high-fat–fed C57BL/6 mice showed reduced osteocalcin expression.

Altogether these results suggest that oxidized lipids adversely affect bone by inhibiting osteoblastic differentiation. If applicable to humans, these studies may result in new therapeutic approaches to osteoporosis.

MATERIALS AND METHODS

Mice and diets

At 1 month of age, male C57BL/6 (atherosclerosis-susceptible strain) and C3H/HeJ (atherosclerosis-resistant strain) mice (The Jackson Laboratory, Bar Harbor, ME, USA) were placed on either a control chow diet (National Institutes of Health [NIH]-31 Mouse/Rat Diet 7013 containing 6% fat) or a high-fat (atherogenic) diet (Teklad TD90221; Harlan Teklad, Madison, WI, USA; including 1.25% cholesterol, 15.8% fat, and 0.5% cholate). This atherogenic diet has been found to cause significant hypercholesterolemia in C57BL/6 mice.[24,25] Femurs and lumbar vertebrae were harvested from 8 animals after 4 months and 14 animals after 7 months. The bones were cleared of soft tissue and fixed in 95% ethanol.

Quantitative computed tomographic scanning

Peripheral quantitative computed tomographic (pQCT) scans were performed on individual bones (left femur, L4 vertebrae) from each mouse. Scanning was done with a STRATEC XCT 960M unit (Norland Medical Instruments, Ft. Atkinson, WI, USA) specifically configured for small bone specimens. Mineral thresholds were set at 1.30 for low-density bone and 2.00 for high-density bone. These thresholds excluded mouse fat, water, muscle, and tendon from true bone. Daily calibration was performed with a manufacturer-supplied phantom (hydroxyapatite in Lucite) of defined density. Calibration with a set of known hydroxyapatite standards (0.05–1000.0 mg/mm^3) yielded a correlation of 0.998 with XCT 960M estimation of volumetric density. Estimates of measurement precision of mineral and volume of femurs and vertebrae were obtained from the middiaphyseal shaft of a B6C3H-F1 femur and from the midbody scans of a B6C3H-F1 L5 vertebra. Six replicate measurements for each bone yielded average values of 1.6, 2.1, and 2.8% for femoral density, mineral, and volume, respectively, and 3.2, 5.9, and 4.7% for L5 vertebral density, mineral, and volume, respectively.

Femurs were scanned full length at 2-mm intervals with a resolution of 0.100 mm/voxel, yielding eight 1-mm-thick cross-sections representing eight axial levels of the femur. Vertebrae were scanned full length at 0.7-mm intervals with the same resolution, yielding three to four 1-mm-thick cross-sections. The center-most scan (based on image morphology) or the mean of two scans sharing the center position was selected for data analyses.

Marrow isolation

After 4 months on the diets, mouse marrow cells were isolated from both femurs from 2 animals in each group as previously described.[21,29,30] Marrow from both femurs was pooled for each animal and RNA was isolated and analyzed separately by reverse-transcriptase polymerase chain reaction (RT-PCR). RNA was isolated as previously described using the RNA isolation kit from Stratagene (La Jolla, CA, USA).[28]

RT-PCR

RNA in 3-μg quantities was reverse-transcribed, and PCR was performed using primers as described previously.[31] Thermal cycling was carried out for 21 cycles (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) or 34 cycles (osteocalcin) at 60°C annealing temperature for both GAPDH and osteocalcin. Amplified fragments were isolated on a 6% polyacrylamide gel (29:1 acrylamide to bis-acrylamide), and the autoradiographs were scanned with an AGFA ARCUS II scanner and semiquantitated with NIH Image software, version 1.59, public domain program (National Institutes of Health, Bethesda, MD, USA).

Lipoprotein preparation and oxidation

Human LDL was isolated by density-gradient centrifugation of serum and stored in phosphate-buffered 0.15 M NaCl containing 0.01% EDTA. MM-LDL was prepared by iron oxidation of human LDL as previously described.[20] Minimal oxidation of LDL resulted in a 2- to 3-fold increase in conjugated dienes and 2–3 nmol of thiobarbituric acid reactive substances per milligram of cholesterol after dialysis. The concentrations of lipoproteins used in this study are reported in micrograms of protein. The pre- and postoxidation lipopolysaccharide levels in these lipoprotein preparations were <30 pg/ml.

Statistical analysis

Differences in BMC and BMD were assessed using Student's two-tailed *t*-test, allowing for unequal variances and unequal sample sizes where appropriate.

TABLE 1. QCT BONE PARAMETERS FOR FEMURS FROM C57BL/6 MICE AFTER 7 MONTHS ON A CONTROL CHOW OR HIGH-FAT DIET

	Mineral content (mg)		*Chow versus high fat*	*Mineral density (mg/mm³)*		*Chow versus high fat*
Slice	*Chow*	*High fat*	p	*Chow*	*High fat*	p
1	2.29 ± 0.82	0.74 ± 0.19	0.002	0.502 ± 0.05	0.441 ± 0.04	0.01
2	1.05 ± 0.21	0.53 ± 0.20	0.001	0.365 ± 0.05	0.355 ± 0.05	0.70
3	1.02 ± 0.08	0.77 ± 0.10	0.0001	0.447 ± 0.05	0.400 ± 0.06	0.02
4	0.99 ± 0.07	0.72 ± 0.06	0.0003	0.440 ± 0.02	0.391 ± 0.02	0.05
5	1.19 ± 0.14	0.78 ± 0.07	<0.0001	0.520 ± 0.04	0.410 ± 0.03	0.01
6	1.26 ± 0.12	0.86 ± 0.10	<0.0001	0.573 ± 0.04	0.465 ± 0.05	<0.0001
7	1.32 ± 0.16	0.75 ± 0.19	0.0008	0.515 ± 0.05	0.416 ± 0.04	0.1
8	1.13 ± 0.43	0.71 ± 0.31	0.001	0.538 ± 0.03	0.476 ± 0.05	0.01
Mean ± SD	1.28 ± 0.42	0.73 ± 0.09	0.008	0.488 ± 0.07	0.419 ± 0.04	0.03

Scans were performed at 8 longitudinal axis positions (slices) for each femur with 1 being most proximal and 8 most distal. Values of BMC and BMD are expressed as mean ± SD over all animals in each diet group.

TABLE 2. QCT BONE PARAMETERS FOR FEMURS FROM C3H/HeJ MICE AFTER 7 MONTHS ON A CONTROL CHOW OR HIGH-FAT DIET

	Mineral content (mg)		*Chow versus high fat*	*Mineral density (mg/mm³)*		*Chow versus high fat*
Slice	*Chow*	*High fat*	p	*Chow*	*High fat*	p
1	2.73 ± 0.79	2.00 ± 0.84	0.12	0.596 ± 0.06	0.542 ± 0.06	0.11
2	1.60 ± 0.24	1.26 ± 0.15	0.01	0.510 ± 0.06	0.426 ± 0.06	0.016
3	1.72 ± 0.16	1.40 ± 0.13	0.002	0.800 ± 0.03	0.720 ± 0.05	0.005
4	1.88 ± 0.24	1.66 ± 0.16	0.07	0.883 ± 0.07	0.909 ± 0.03	0.39
5	2.01 ± 0.18	1.73 ± 0.16	0.009	0.853 ± 0.03	0.846 ± 0.02	0.63
6	2.28 ± 0.27	2.06 ± 0.14	0.09	0.922 ± 0.06	0.911 ± 0.02	0.68
7	2.01 ± 0.41	1.95 ± 0.15	0.75	0.694 ± 0.11	0.807 ± 0.09	0.05
8	1.47 ± 0.40	1.55 ± 0.21	0.67	0.612 ± 0.07	0.562 ± 0.04	0.15
Mean ± SD	1.96 ± 0.40	1.70 ± 0.29	0.59	0.734 ± 0.15	0.715 ± 0.19	0.26

Scans were performed at 8 longitudinal axis positions (slices) for each femur with 1 being most proximal and 8 most distal. Values of BMC and BMD are expressed as mean ± SD over all animals in each diet group.

RESULTS

Femoral BMC and BMD

After 4 months, femoral BMD was significantly lower in fat-fed C57BL/6 mice at three of the eight levels scanned ($p < 0.04$; from 0.488 ± 0.038 mg/mm³ to 0.423 ± 0.043 mg/mm³). All three levels were in the middiaphyseal region where variance caused by anatomic complexity is minimized. BMC was not significantly different between the two groups.

After 7 months, femoral BMC was significantly lower in fat-fed C57BL/6 mice compared with control chow-fed mice at all eight levels scanned. Mean mineral content was lowered 43% (from 1.28 ± 0.42 mg to 0.73 ± 0.09 mg; $p \leq 0.002$; Table 1) on the high-fat diet. Changes in mineral content were most significant ($p \leq 0.0003$) at the four middiaphyseal levels (scans 3–6). Femoral mineral density was also significantly lower in fat-fed C57BL/6 mice compared with chow-fed mice at six of eight levels, with a 14.5% mean difference (from 0.488 ± 0.066 mg/mm³ to 0.419 ± 0.035 mg/mm³; $p = 0.03$; Table 1).

In C3H/HeJ mice, which are resistant to the atherogenic effects of a high-fat diet and lipid oxidation products,[24,25] the high-fat diet had less effect on bone mineralization. After 4 months on the diet, C3H/HeJ mice showed no significant difference in femoral BMC at any of the eight levels examined (data not shown); BMD was significantly lower at one of eight scanned sites ($p = 0.01$).

After 7 months on the diet, the fat-fed C3H/HeJ mice had significantly ($p \leq 0.01$) lower BMC compared with chow-fed mice at only three of eight levels (Table 2). However, the overall mean difference for all eight levels did not reach statistical significance ($p = 0.59$). There also was no significant effect of the high-fat diet on femoral mineral density ($p = 0.26$; Table 2).

Lumbar vertebral mineral content and mineral density

At 4 months, there was no significant difference between chow and high-fat diet groups in either vertebral mineral content or density in either mouse strains. However, at 7

TABLE 3. QCT BONE PARAMETERS FOR L4 VERTEBRAE FROM C57BL/6 AND C3H/HeJ MICE AFTER 7 MONTHS ON A CONTROL CHOW OR HIGH-FAT DIET

Total bone				*Cortical bone*			
Mineral content (mg)		*Mineral density (mg/mm³)*		*Mineral content (mg)*		*Mineral density (mg/mm³)*	
Chow	*High fat*	*Chow*	*High fat*	*Chow*	*High fat*	*Chow*	*High fat*
			C57BL/6				
1.20 ± 0.10	0.77 ± 0.10	0.229 ± 0.02	0.212 ± 0.03	0.317 ± 0.08	0.088 ± 0.05	0.455 ± 0.01	0.445 ± 0.01
p	<0.001		0.30		<0.001		0.09
			C3H/HeJ				
1.41 ± 0.35	1.31 ± 0.14	0.248 ± 0.03	0.217 ± 0.01	0.624 ± 0.25	0.445 ± 0.13	0.481 ± 0.02	0.474 ± 0.01
p	0.49		0.03		0.12		0.48

Values are for the central slice or slices for each of the L4 vertebrae and are expressed as mean ± SD over all animals in each diet group.

months, vertebral mineral content was significantly lower in the C57BL/6 fat-fed mice (Table 3). Total mineral content of the central section or sections was lower by a mean of 35% (from 1.2 ± 0.1 mg to 0.77 ± 0.1 mg; $p < 0.001$), primarily because of changes in high-density cortical bone (i.e., a 72% decrease). Total mineral density decreased 7% on the high-fat diet, but this change was not statistically significant. In C3H/HeJ mice, a 7% decrease in total mineral content was found, as well as a 29% decrease in cortical mineral content. These changes did not reach statistical significance. Total mineral density of vertebrae from C3H/HeJ mice decreased 12.5% on the high-fat diet (from 0.248 ± 0.03 to 0.217 ± 0.01 mg/mm³; $p = 0.03$).

Gene expression in marrow cells

After 4 months on the high-fat or chow diets, the marrow isolated from 2 C57BL/6 mice on each diet was analyzed for the expression of three markers of osteoblastic differentiation: alkaline phosphatase, bone sialoprotein, and osteocalcin. All three markers were expressed by the marrow cells. Of the three, only osteocalcin expression was affected by diet, showing a 35% reduction with the high-fat diet when normalized to GAPDH values (Fig. 1).

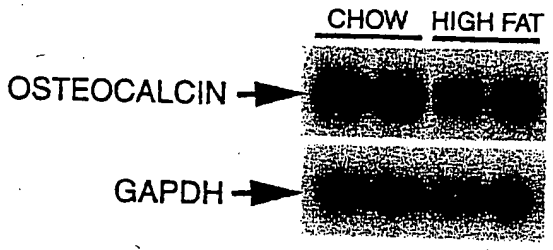

FIG. 1. Effects of a high-fat diet on osteocalcin expression in marrow cells. One-month-old C57BL/6 mice were placed on a high-fat or chow diet for 4 months. The animals were killed and femoral marrow was isolated from each mouse and used to isolate total RNA. RT-PCR analysis showed an expected size band of 360 base pairs (bp). Expression of GAPDH was used for normalization. Each lane represents RNA isolated from an individual mouse.

DISCUSSION

The present study is the first to show that 7-month treatment with an atherogenic high-fat diet lowers BMD and BMC in vivo in atherosclerosis-susceptible C57BL/6 mice, with much smaller effects in the atherosclerosis-resistant C3H/HeJ mice. The atherogenic diet resulted in a significantly lower femoral mineral content and femoral mineral density in the C57BL/6 mice. Smaller changes were seen in the C3H/HeJ mice. The differential effects of the atherogenic diet on bones in the two strains of mice are similar to the effects of that diet on the development of atherosclerosis. Previous reports showed differences in genetically determined factors in response to diet-induced hyperlipidemia and lipid oxidation in these mouse strains to be the underlying reason for their degree of susceptibility to atherosclerosis. These differences include: (1) the level of induction of inflammatory genes such as monocyte chemotactic protein-1, colony-stimulating factors, heme oxygenase, and serum amyloid A and activation of nuclear factor κB (NFκB) transcription factor in response to atherogenic diet[26,27,32] and (2) the ability of high-density lipoprotein (HDL) to protect against the effects of atherogenic diet, because of variability in the level of antioxidant enzyme paraoxonase.[28] The latter difference is important in light of the observation that the protective effect of HDL appears to correlate inversely with atherosclerosis,[33] and a direct correlation between HDL levels and BMD in fat-fed mice has been shown (T. Drake, University of California, Los Angeles [UCLA], Department of Pathology, personal communication, 1999). It is intriguing to speculate that similar genetically regulated factors, involved with defense against atherogenic oxidized lipids, also determine susceptibility to osteoporosis.

Because femoral mineral content was more substantially changed by the atherogenic diet than mineral density, the effect may be caused by quantitatively less bone formation

and/or shorter bones in the high-fat–fed mice. Although we did not measure femoral size after 7 months in this study, in a separate study, we found no significant change in the femoral or tibial length between chow-fed versus high-fat–fed C57BL/6 mice after 4 months on the diet (F. Parhami, unpublished observations, 1999). Because our previous in vitro and in vivo studies showed inhibition of osteoblastic differentiation and bone formation by marrow stromal cells isolated from C57BL/6 mice on the high-fat diet versus chow diet, we speculate that bone formation is inhibited by the atherogenic diet. More direct future studies will further validate this speculation. It is important to note that the mice used in the present study were in their growing stage when peak bone mass is achieved. Inhibition of bone formation during growth stage also would have adverse consequences by reducing peak bone mass. The reducing effects of the dietary fat on BMC and BMD would translate into a reduction in this important determinant of bone strength.

The present results also suggest that increased dietary lipids interfere with osteoblast maturation in vivo, based on dietary inhibition of osteocalcin messenger RNA (mRNA) expression. Although the effect of the high-fat diet on the expression of osteocalcin alone is not sufficient to draw definitive conclusions about differentiation of osteoblasts, this inhibition is consistent with previous ex vivo evidence that exposure to a high-fat diet reduced marrow preosteoblastic maturation in culture,[21] as well as in vitro evidence that lipid and lipoprotein oxidation products inhibit osteoblast differentiation and function.[20,21] Previous studies using the same atherogenic diet in C57BL/6 mice have shown 2- to 3-fold increases in cholesterol levels after 3–4 weeks on this diet, as well as a significant drop in the HDL levels.[24,25] We therefore speculate that the adverse effects of the high-fat diet on bone in the C57BL/6 mice are caused by dyslipidemia and subsequent increases in lipid oxidation. The diet-induced hyperlipidemia in circulation further translates into increased lipid accumulation in highly vascular tissues and the artery wall because of the diffusion of lipoproteins across the vascular endothelium. Once apart from the protective, antioxidant environment of serum, these lipoprotein particles are oxidized further into biologically active forms responsible for inflammatory processes in atherosclerosis and vascular calcification.[19,20] Because bone and marrow are both vascularized, circulating lipids can access both sites of active bone remodeling where osteoprogenitor cells are present: (1) the subendothelial space of the osteons and (2) the marrow stroma at the trabecular surface or endosteum. Lipid accumulation[34] and monocyte accumulation and plaquing[35] have been observed in the vessels of osteons in osteoporotic and aging bone. The presence of circulating lipoproteins in the marrow is expected because marrow is a site for clearance of chylomicrons and chylomicron remnants derived from dietary fat,[36] and dietary fat has been found to alter the lipid profile in the marrow.[37] Thus, lipid oxidation products may underlie the paradoxical association of cardiovascular disease with osteoporosis.

The findings in the present report are consistent with a preliminary report showing a significant correlation between dietary cholesterol intake and vertebral bone loss in women,[38] as well as with population studies showing an association of cholesterol levels with osteoporosis in women[13] and, preliminarily, in men.[39] Recent evidence suggests that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), lipid-lowering agents commonly used to treat cardiovascular disease, have potent positive effects on bone formation in rodents,[40] and statin therapy in humans correlates with reduced osteoporosis.[41–43] Although the mechanism is proposed to be a direct stimulation of osteoblasts, an equally likely mechanism is an indirect effect through lipid-lowering, given that the dominant site of action of these agents, in both humans and rodents,[46] is in the liver where statins are mostly cleared from circulation.

Evidence suggests that the atherogenic nature of the high-fat diet is essential for effects on bone. Wohl et al. previously showed a minimal effect on BMC of a noncholesterol, 8% fat diet in adult roosters.[47] Because cholesterol feeding is necessary to induce atherosclerosis in roosters,[48] this finding suggests that a nonatherogenic high-fat diet is not sufficient to induce bone changes.

Collectively, these observations suggest the adverse effects of lipids on bone. The possibility that lipid oxidation products are the biologically active factors linking a high-fat diet with reduced bone formation is supported by the finding of substantially reduced effects in mice that are resistant to the effects of oxidized lipids and by the anabolic effects of the antioxidant vitamin E on bone.[49] Because cardiovascular disease is the highest risk cause of death for patients with osteoporotic fracture[4,5] and low BMD is associated with mortality independent of fractures,[50] elucidation of common lipid- and lipid oxidation–mediated mechanisms has great importance for identifying new preventive measures for both osteoporosis and cardiovascular disease. The possibility that high lipid levels are a common underlying factor in atherosclerosis and bone loss may explain the epidemiological evidence for correlation between cardiovascular disease and osteoporosis.

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Low-density lipoprotein receptor-related protein 5 (LRP5) is essential for normal cholesterol metabolism and glucose-induced insulin secretion

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A Wnt coreceptor low-density lipoprotein receptor-related protein 5 (LRP5) plays an essential role in bone accrual and eye development. Here, we show that LRP5 is also required for normal cholesterol and glucose metabolism. The production of mice lacking LRP5 revealed that LRP5 deficiency led to increased plasma cholesterol levels in mice fed a high-fat diet, because of the decreased hepatic clearance of chylomicron remnants. In addition, when fed a normal diet, LRP5-deficient mice showed a markedly impaired glucose tolerance. The LRP5-deficient islets had a marked reduction in the levels of intracellular ATP and Ca^{2+} in response to glucose, and thereby glucose-induced insulin secretion was decreased. The intracellular inositol 1,4,5-trisphosphate (IP3) production in response to glucose was also reduced in LRP5−/− islets. Real-time PCR analysis revealed a marked reduction of various transcripts for genes involved in glucose sensing in LRP5−/− islets. Furthermore, exposure of LRP5+/+ islets to Wnt-3a and Wnt-5a stimulates glucose-induced insulin secretion and this stimulation was blocked by the addition of a soluble form of Wnt receptor, secreted Frizzled-related protein-1. In contrast, LRP5-deficient islets lacked the Wnt-3a-stimulated insulin secretion. These data suggest that Wnt/LRP5 signaling contributes to the glucose-induced insulin secretion in the islets.

diabetes | Wnt protein | chylomicron remnant | pancreatic β cells | insulin-like growth factor 1

Low-density lipoprotein (LDL) receptor-related protein (LRP)5 and LRP6 are coreceptors involved in the Wnt signaling pathway (1–6). The Wnt signaling pathway plays a pivotal role in embryonic development (7, 8) and oncogenesis (9) through various signaling molecules including Frizzled receptors (10), recently characterized LRP5 and LRP6 (1–6), and Dickkopf proteins (4, 6). In addition, the Wnt signaling is also involved in adipogenesis by negatively regulating adipogenic transcription factors (Tcfs) (11). Although Wnt signaling has been characterized in both developmental and oncogenic processes, little is known about its function in the normal adult.

Recent studies have revealed that loss of function mutations in the LRP5 gene cause the autosomal recessive disorder osteoporosis–pseudoglioma syndrome (12). LRP5 is expressed in osteoblasts and transduces Wnt signaling via the canonical pathway, thereby modulating bone accrual development (12, 13). A point mutation in a "propeller" motif in LRP5 causes a dominant-positive high bone density by impairing the action of a normal antagonist of the Wnt pathway, Dickkopf, thereby increasing Wnt signaling (14, 15). In addition, the human LRP5 gene is mapped within the region (IDDM4) linked to type 1 diabetes on chromosome 11q13 (16).

In previous studies, we and others showed that LRP5 is highly expressed in many tissues, including hepatocytes and pancreatic beta cells (17, 18). We also showed that LRP5 can bind apolipoprotein E (apoE) (18). This finding raises the possibility that LRP5 plays a role in the hepatic clearance of apoE-containing chylomicron remnants, a major plasma lipoprotein carrying diet-derived cholesterol.

To evaluate the *in vivo* roles of LRP5, we generated LRP5-deficient mice. In this paper, we describe a function of LRP5 in the metabolism of cholesterol and glucose. Our data indicate that LRP5 is a multifunctional receptor involved in multiple pathways, including bone development, cholesterol metabolism, and the modulation of glucose-induced insulin secretion.

Experimental Procedures

Generation of LRP5-Deficient Mice. To produce mice carrying a mutated LRP5 gene, a targeting vector was constructed from a genomic DNA fragment containing exons 17 and 18 of the murine LRP5 gene. A neomycin-resistance gene under transcriptional control of the mouse phosphoglycerate kinase-1 promoter (PGK-neo) was inserted into the *Xho*I site within exon 18 of the mouse LRP5 gene. The 5′ and 3′ DNA fragments flanking PGK-neo were ligated into a pMCDT-A plasmid (GIBCO/BRL) composed of a poly(A)-less *neo* gene, a polymerase destabilizing signal, a pausing signal for RNA polymerase II, and the diphtheria toxin A fragment (DT-A) gene for negative selection (19). TT2 embryonic stem (ES) cells (20) were

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Abbreviations: apoE, apolipoprotein E; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CM, conditioned medium; HNF, hepatocyte nuclear factor; IGF, insulin-like growth factor; IRS, insulin receptor substrate; IP3, inositol 1,4,5-trisphosphate; LDL, low-density lipoprotein; LRP, LDL receptor-related protein; sFRP-1, secreted Frizzled-related protein-1; Tcf, transcription factor.

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transfected using standard techniques (21). Chimeric males were generated using the morula aggregation technique, and mated to C57BL/6J female mice. After achieving germ-line transmission, LRP5+/− females were crossed with C57BL/6J males. For immunoblotting, an antibody against murine LRP5 peptide (ATLYPPILNPPPSPA, amino acids 1490–1504, NCBI Protein database accession no. NP_032539) was generated. The antibody binding was detected with a chemiluminescence detection kit (ECL, no. RPN2106, Amersham Pharmacia Biotech).

Plasma Clearance and Hepatic Uptake of Chylomicron Remnants. Chylomicron remnants were prepared using a modified method of Redgrave and Martin (22) using functionally hepatectomized rats, and labeled with fluorescent lipid (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, DiI) as described by Takahashi *et al.* (23). Mice fed a high-fat diet for 16 weeks were injected i.v. into a femoral vein, with fluorescent chylomicron remnants from rat (5 μg per mouse) in 0.2 ml of PBS. Blood was sampled at various times and, after extracting lipid, plasma fluorescence was measured with a spectrofluorometer. The amount of fluorescence remaining in the plasma is expressed as a percentage of the calculated initial blood concentration, assuming that plasma volume is 4.4% (vol/wt) of body weight. After collection of the final blood samples, the mice were exsanguinated and livers were excised for the extraction of lipids and measurement of fluorescence.

Blood Glucose and Serum Insulin. Mice (6–8 months old) were fasted for 12 h and then given an i.p. injection of glucose (1 g/kg of body weight). Blood samples were obtained from the tail vein at the indicated times after the glucose load. Blood glucose and plasma insulin levels were measured with the Glucose CII test Wako (Wako Pure Chemical, Osaka) and an insulin RIA kit (Shionogi, Osaka), respectively.

Analysis of Pancreatic Islets. The procedure for the isolation of pancreatic islets is described in *Supporting Methods*, which is published as supporting information on the PNAS web site, www.pnas.org. For the measurement of insulin secretion from islets, pancreatic islets from 6- to 8-month-old mice were pooled and cultured in RPMI medium 1640 containing 11.6 mM glucose, 1% penicillin–streptomycin, 10% FBS, and 25 mM Hepes at pH 7.4 (medium A). Pancreatic islets cells were infected with recombinant adenoviruses encoding LRP5 (AdLRP5) (18) or LacZ (AdLacZ) according to the procedure by Becker *et al.* (24). After culturing for 16–20 h, islets were transferred to Krebs–Ringer buffer (KRB; Sigma) containing 0.2% BSA for the measurement of insulin secretion studies by using an RIA kit (Amersham Pharmacia Biotech). For measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), pancreatic islet cells were cultured on a collagen-coated, glass-bottomed well for 16 h in medium A, and loaded with the fluorescent Ca^{2+} indicator Fluo3/AM as described by Katoh *et al.* (25). Changes in $[Ca^{2+}]_i$ were measured using a confocal laser scanning microscope (Axiovert 100, Zeiss) with a ×40 objective lens. Intracellular levels of ATP and ADP were measured with a luciferase–luciferin system by using an ATP determination kit (catalog number A-22066, Molecular Probes; ref. 26). Intracellular levels of inositol 1,4,5-trisphosphate (IP3) were determined using an IP3 RIA kit (Amersham Pharmacia Biotech).

Wnt-Conditioned Media (CM) and Purified Secreted Frizzled-Related Protein-1 (sFRP-1). CM from Wnt-3a, Wnt-5a, and parental vector-transfected L cells were prepared according to Shibamoto *et al.* (27). CM were diluted 5-fold with medium A. After incubation with CM for 16 h, islets were transferred to Krebs–Ringer buffer for the measurement of insulin for secretion studies.

An expression plasmid encoding recombinant sFRP-1 containing Myc/polyhistidine epitopes (28) was used to produce recombinant sFRP-1 in COS7 cells. COS7 cells were transfected with the expression plasmid by using the Lipofectamine reagent (GIBCO/BRL). Twenty-four hours after the transfection, the cells were switched to a serum-free medium (OPTI-PRO, GIBCO/BRL) and cultured for 48 h. Recombinant sFRP-1 was purified from the culture medium of transfected cells by using a HisTrap kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol. The purity of the purified protein was verified by immunoblot analysis with anti-Myc tag antibody (Cell Signaling Technology, Beverly, MA).

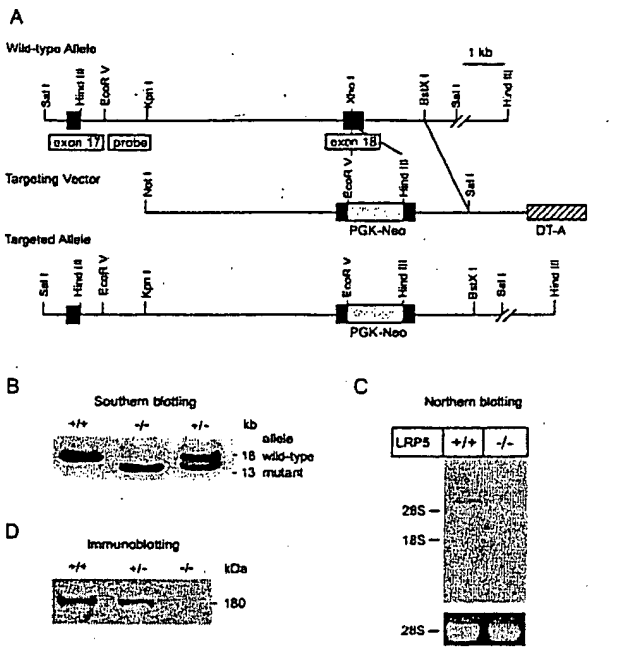

Fig. 1. Generation of LRP5-deficient mice. (*A*) Diagram of the targeting strategy. Only the relevant restriction sites are indicated. (*B*) Southern blot analysis of *Hin*dIII-digested DNA from LRP5+/+, LRP5−/−, and LRP5+/− mice. Southern blotting was performed with the probe indicated in *A*. *Hin*dIII digestion resulted in an 18-kb fragment in wild-type DNA and a 13-kb fragment in homologous recombinants. A typical autoradiogram is shown. (*C*) Northern blot analysis of LRP5 transcripts. Total RNA (15 μg) from the livers of LRP5+/+ and LRP5−/− mice was hybridized with a mouse LRP5 cDNA probe (extended from nucleotide 2401 to nucleotide 2991). A typical autoradiogram (48-h exposure) is shown. RNA loading was consistent among the lanes as judged by ethidium bromide staining and reprobing with glyceraldehyde-3-phosphate dehydrogenase. (*D*) Immunoblot analysis, using an anti-mouse LRP5 antibody, of LRP5+/+, LRP5+/−, and LRP5−/− mouse liver membrane fractions. Each lane was loaded with 500 μg of crude membrane fraction from the liver homogenates. Protein loading was consistent among the lanes as judged by Ponceau staining.

Results

Generation of LRP5-Deficient Mice. An insertion-type vector was constructed to disrupt an exon encoding a ligand-binding repeat of the mouse LRP5 gene (exon 18; Fig. 1*A*). Three lines of mice lacking LRP5 were identified by Southern blotting (Fig. 1*B*), and the absence of LRP5 transcripts (Fig. 1*C*) and protein (Fig. 1*D*) in the liver was confirmed by Northern blot and immunoblot analyses, respectively.

Wild-type (LRP5+/+), heterozygous (LRP5+/−), and homozygous (LRP5−/−) mice were born with frequencies predicted by simple Mendelian ratios. In contrast to the severe developmental defects of LRP6 mutant mice (3), LRP5−/− mice of both sexes developed and appeared normal, gaining weight at a rate equal to that of LRP5+/+ mice and were normally fertile. Under light-

microscopic examination of LRP5-deficient males, there were no apparent histological abnormalities in the tissues examined, including bone, brain, eye, kidney, liver, and pancreas.

Although no apparent low-bone-mass phenotype was observed in 3- to 6-month-old LRP5−/− males under light-microscopic examination, we noticed that the femur and parietal bones were thin and fragile in LRP5−/− females older than 6 months. The thickness of the parietal portion of calvaria of LRP5−/− mice was significantly reduced to 50–60% of the controls ($n = 3$, $P < 0.04$; Fig. 6, which is published as supporting information on the PNAS web site). Similarly, the thickness of tibias of LRP5−/− females was also reduced to 60–70% of the controls (data not shown). We also found some cases of pathological fracture of lower limbs in these mice. Recently, Kato *et al.* (13) generated LRP5−/− mice developing a severe low-bone-mass phenotype similar to that of patients with osteoporosis-pseudoglioma syndrome. The low-bone-mass phenotype of LRP5−/− generated by Kato *et al.* was observed regardless of sex and age, and a significant number of the mice died within the first month of life because of fractures. The relatively modest bone phenotype of our LRP5−/− females resembles the osteoporosis of humans and suggests that the involvement of other factors, including sex, aging, hormonal status, dietary exposure, and genetic background in the development of a low-bone-mass phenotype.

Impaired Chylomicron Clearance. To determine the metabolic consequences of LRP5 deficiency, we analyzed the effects of LRP5 deficiency on lipoprotein metabolism by using LRP5−/−, +/−, and +/+ mice. The plasma levels of cholesterol in LRP5+/− and LRP5−/− mice that were fed a standard laboratory chow were identical to those of their LRP5+/+ littermates (Fig. 7, which is published as supporting information on the PNAS web site). In contrast, when mice were fed a high-fat diet containing 7.5% coconut oil and 1.25% cholesterol, plasma cholesterol levels were significantly increased in both LRP5+/− and LRP5−/− mice. The levels of plasma cholesterol in LRP5−/− mice fed a high-fat diet for 2 months were ≈200 mg/dl, whereas those in LRP5+/+ littermates were ≈170 mg/dl (Fig. 2*A*). HPLC analysis of the plasma lipoprotein profile revealed that very low-density lipoprotein cholesterol was increased in LRP5−/− mice after being fed a high-fat diet (Fig. 7). The levels of plasma triglyceride in LRP5+/+ and −/− mice were indistinguishable (within the range of 50–80 mg/dl).

apoE-containing chylomicron remnants can be cleared normally in LDL receptor-lacking familial hypercholesterolemia patients and Watanabe hereditary hyperlipidemic rabbits (29). To determine the effects of LRP5 deficiency on the plasma clearance of chylomicron remnants, we injected fluorescently labeled chylomicron remnants into LRP5−/− mice and +/+ littermates fed a high-fat diet. As shown in Fig. 2*B*, approximately half of the injected chylomicron remnants were cleared from the plasma of LRP5+/+ mice at 30 min after injection, whereas >80% remained in the plasma of LRP5−/− mice. Consistent with the delayed clearance, hepatic uptake of the injected fluorescence was markedly reduced in LRP5−/− mice (≈16% of LRP5+/+ mice, Fig. 2*C*). A similar result was obtained for apoE-rich β-migrating very low-density lipoprotein (data not shown). The mRNA levels of LDL receptor and LRP1 (a candidate chylomicron remnant receptor; ref. 30), were indistinguishable between LRP5+/+ and −/− mice (data not shown). These data indicate that LRP5 recognizes apoE-containing lipoproteins *in vivo* and plays a role in the hepatic clearance of chylomicron remnants.

Impaired Glucose-Induced Insulin Secretion. We next analyzed the effects of LRP5 deficiency on glucose metabolism in LRP5−/− mice. Mice were fed either a normal laboratory chow diet (CE-2, CLEA Japan, Osaka) or a high-fat diet containing 1.5% cholesterol, 7.5% olive oil, 5% cholic acid, and 7.5% milk casein in a standard

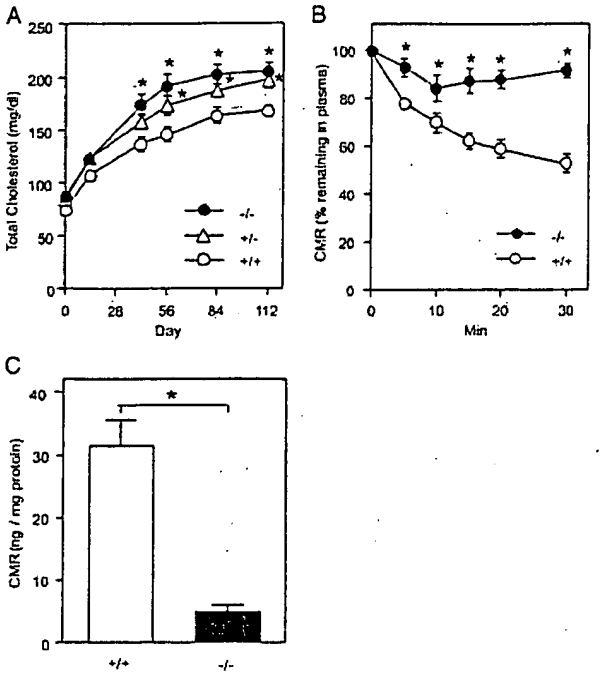

Fig. 2. Diet-induced hypercholesterolemia in LRP5-deficient mice. (*A*) Total plasma cholesterol levels in mice that were fed a high-fat diet. Mice (7–8 weeks of age) heterozygous (LRP5+/−) and homozygous (LRP5−/−) for LRP5 deficiency and their wild-type littermates (LRP5+/+) were fed a high-fat diet for 16 weeks, during which plasma total cholesterol levels of each mouse were measured at the indicated times. The values are the mean ± SE for six mice. *, $P < 0.05$ compared with LRP5+/+. (*B* and *C*) Plasma clearance (*B*) and liver uptake (*C*) of injected chylomicron remnants (CMR). The values are the mean ± SE for six mice. *, $P < 0.01$; Student's *t* test.

laboratory chow diet. Although fasted blood glucose and insulin levels in LRP5−/− and +/− mice appeared identical to those of their +/+ littermates, even after being fed a high-fat diet (90–110 mg/dl), LRP5−/− and LRP5+/− mice exhibited impaired glucose tolerance (IGT) during an i.p. glucose-tolerance test (Fig. 3*A*). This IGT was observed regardless of sex; however, it was age-dependent, because significant glucose intolerance was not seen in LRP5-deficient mice before 6 months of age. Consistent with the marked glucose intolerance, the glucose-induced increase in plasma insulin concentration was lower in both LRP5−/− and LRP5+/− mice than in LRP5+/+ mice (Fig. 3*B*). Pancreatic sections from 6-month-old LRP5+/+ and LRP5−/− mice showed no manifestation of insulitis, including the infiltration of lymphocytes or reduced cell mass in LRP5−/− islets (Fig. 8*A*, which is published as supporting information on the PNAS web site). Similarly, the appearances of alpha and beta cells of the islets were almost indistinguishable in LRP5+/+ and −/− mice as determined by Grimelius (alpha cells; Fig. 8*B*) and aldehyde–fuchsin staining (beta cells; Fig. 8*C*). Pancreatic insulin levels in LRP5−/− mice were not significantly different from those of LRP5+/+ mice and are as follows: 7.07 ± 0.94 and 6.40 ± 0.48 milliunits/mg of protein in LRP5+/+ and LRP5−/− mice, respectively ($n = 6$; Fig. 8*D*). Pancreas weights were also indistinguishable in LRP5+/+ and −/− mice (306 ± 17 and 296 ± 8 mg in LRP5+/+ and LRP5−/− mice, respectively, $n = 6$). No apparent differences were seen in the size of the islets between LRP5+/+ and −/− mice either, as shown in Table 3, which is published as supporting information on the PNAS web site. An i.p. insulin tolerance test revealed that LRP5−/− mice fed a normal diet were not insulin resistant (data not shown). In contrast to LRP5−/− mice, IGT was not seen in mice lacking apoE (data not shown), suggesting that the IGT in

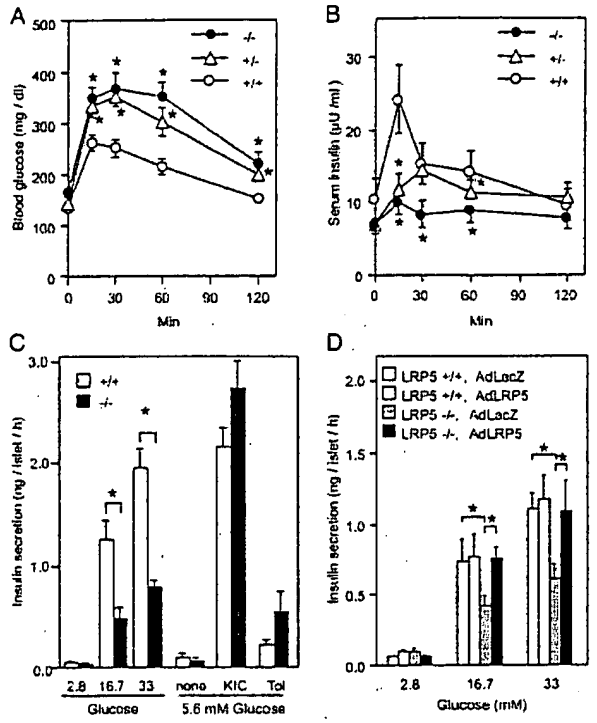

Fig. 3. Impaired glucose-induced insulin secretion in LRP-deficient mice and amelioration by AdLRP5. (*A* and *B*) Blood glucose (*A*) and serum insulin (*B*) levels in LRP5+/+, +/−, and −/− mice after glucose injection. (*C*) Impaired insulin secretion from the islets of LRP5−/− mice. Insulin secretion was induced by different concentrations of glucose and 0.2 mM tolbutamide (Tol), or 10 mM α-ketoisocaproate (KIC), in the presence of 5.6 mM glucose. (*D*) Restoration of insulin secretion by AdLRP5. Pancreatic islets were isolated from LRP5+/+ and −/− mice, infected with recombinant adenoviruses encoding LRP5 (AdLRP5) or LacZ (AdLacZ), and insulin secretion was measured at various glucose concentrations. The values in *A* and *B* are the mean ± SE for six mice; those in *C* and *D* are the mean ± SE for four mice. *, $P < 0.01$; Student's *t* test.

LRP5+/− and −/− mice is independent of apoE binding to LRP5. Although LRP5−/− islets showed impaired glucose-induced insulin secretion, no apparent insulin resistance was observed in LRP5−/− mice. Other factors, including aging, obesity, and prolonged high-fat feeding, may therefore be required to induce typical type 2 diabetes in LRP5−/− mice.

To further define the effects of LRP5 deficiency on glucose-induced insulin secretion, pancreatic islets were prepared from LRP5+/+ and −/− mice, and the changes in the levels of glucose-induced insulin secretion were analyzed. Consistent with the glucose-tolerance test, the change in the insulin secretory response to glucose in LRP5−/− islets was profoundly lower than that of LRP5+/+ islets, particularly at higher concentrations (Fig. 3*C*). When islets were incubated with 10 mM α-ketoisocaproate, which is used for ATP production (31), the changes in the levels of insulin secretion from islets of LRP5−/− mice were approximately the same as those from LRP5+/+ mice, suggesting that there is no impaired ATP production from α-ketoisocaproate in the mitochondrial tricarboxylic acid cycle. Similarly, when cells were incubated with 0.2 mM tolbutamide, there were no changes in the levels of insulin secretion in LRP5−/− and +/+ mice. To restore the impaired insulin secretory response to glucose, LRP5−/− islets were infected with recombinant adenovirus encoding human LRP5 (AdLRP5). As shown in Fig. 3*D*, infection of LRP5−/− islets with AdLRP5 caused their glucose-induced insulin secretion to recover to the levels of LRP5+/+ islets infected with control adenovirus encoding β-galactosidase (AdLacZ) or AdLRP5.

To further evaluate the impaired glucose-induced insulin secretion in LRP5−/− islets, we compared ATP and ADP levels in the islets. As shown in Table 1, the ATP content and ATP/ADP ratio in the presence of 22.2 mM glucose were significantly decreased (by 25–30%) in LRP5−/− islets compared with those in +/+ islets. In contrast, the glycogen content of the islets and hepatic glycogen synthase activity were almost unaltered in LRP5−/− mice (data not shown). Taken together, these data (Fig. 3*C* and Table 1) suggest that the glycolytic pathway was impaired in the LRP5−/− islets.

Consistent with the decreased ATP content and ATP/ADP ratio, glucose-induced intracellular $[Ca^{2+}]_i$ level was markedly decreased in uninfected LRP5-deficient islets. Fig. 4 *A* and *B* shows the glucose-induced $[Ca^{2+}]_i$ increase (as determined by changes in fluorescence intensity) in LRP5+/+ and −/−islets infected with AdLacZ or AdLPR5. The glucose-induced $[Ca^{2+}]_i$ increase in LRP5−/− islets infected with AdLacZ (Fig. 4*B*) was markedly lower than that of LRP5+/+ islets infected with AdLacZ or AdLRP5 (Fig. 4*A*). The average changes in fluorescence intensity (in arbitrary units) by glucose (2.8 mM → 20 mM) in LRP5−/− and LRP5+/+ islets infected with AdLacZ were 29.85 ± 4.04 and 11.10 ± 1.36, respectively ($n = 30$; $P < 0.001$). When LRP5-deficient islets were infected with AdLRP5, the glucose-induced $[Ca^{2+}]_i$ was restored almost completely to normal levels (Fig. 4*B*). There were no statistical differences in the increases in $[Ca^{2+}]_i$ among LRP5+/+ islets infected with AdLacZ or AdLRP5 and LRP5−/−islets infected with AdLRP5. We also examined the glucose-induced production of IP3. The glucose-induced intracellular levels of IP3 were profoundly reduced in LRP5−/− islets (Fig. 4*C*). When LRP5-deficient islets were infected with AdLRP5, the glucose-induced IP3 production was restored almost completely to normal levels (Fig. 4*D*).

Real-Time PCR Analysis. To identify the mechanism underlying the impaired glucose-stimulated insulin secretion, especially the reduced glycolytic pathway, we evaluated steady-state mRNA levels of glucose-sensing proteins and the hepatocyte nuclear factor (HNF) family of transcriptional factors by real-time PCR (see *Supporting Methods*). As shown in Table 2, the mRNA levels of insulin-like growth factor (IGF)-1 receptor, insulin receptor

Table 1. ATP and ADP contents and the ATP/ADP ratio in glucose-stimulated islets

	LRP5+/+		LRP5−/−	
Measurement	2.8 mM glucose	22.2 mM glucose	2.8 mM glucose	22.2 mM glucose
ATP, pmol/islet	4.69 ± 0.08	9.95 ± 0.78	5.20 ± 0.16	7.91 ± 0.74*
ADP, pmol/islet	2.66 ± 0.23	1.89 ± 0.07	1.93 ± 0.15	1.81 ± 0.10
ATP/ADP ratio	2.10 ± 0.23	4.93 ± 0.07	2.31 ± 0.12	3.37 ± 0.20**

Islets of four mice were pooled and incubated at 37°C in the presence of 2.8 or 22.2 mM glucose. After 1 h, the incubation was stopped by the addition of 0.125 ml of trichloroacetic acid to a final concentration of 5%. The islets were disrupted by sonication, and ATP and ADP contents were measured with a luciferase–luciferin system (25). Data are from four independent experiments. Each value represents the mean ± SE. *, $P < 0.01$; **, $P < 0.001$; as compared with wild-type islets (one-way ANOVA).

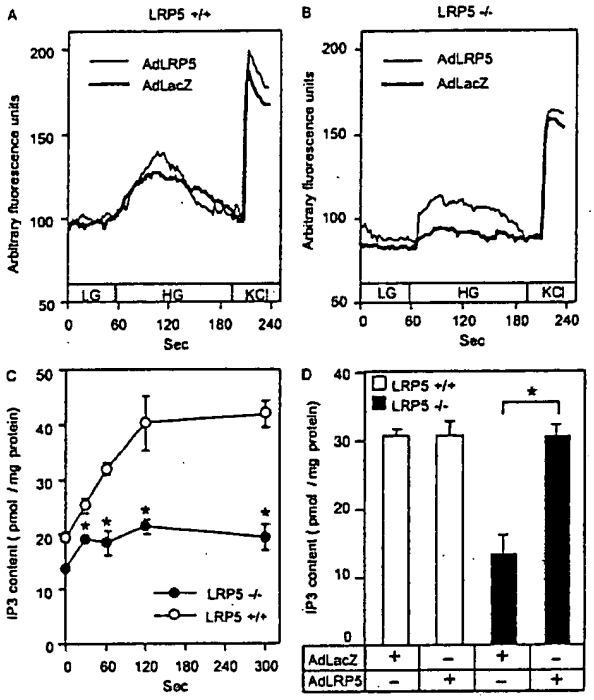

Fig. 4. Impaired glucose-induced $[Ca^{2+}]_i$ increase and IP3 production in LRP5-deficient islets. (*A* and *B*) LRP5+/+ (*A*) and −/− (*B*) islets infected with AdLRP5 or control AdLacZ. Changes in $[Ca^{2+}]_i$ were measured under low glucose (2.8 mM; LG), high glucose (20 mM; HG), or 20 mM KCl (KCl). Representative data from 30 experiments are shown. (*C*) Time course of IP3 content in response to 20 mM glucose in LRP5+/+ and −/− islets. Values are the mean ± SE from quadruplicate determinations. (*D*) Restoration of IP3 production by AdLRP5. Pancreatic islet cells were isolated from LRP5+/+ and −/− mice and infected with AdLRP5 or AdLacZ, and the IP3 content was measured at 5 min after exposure to 20 mM glucose. The values are the mean ± SE from quadruplicate determinations. *, $P < 0.01$; Student's *t* test.

substrate-2 (IRS-2), HNF-4α, insulin receptor, and Tcf1 (HNF-1α) transcripts were drastically decreased in LRP5−/− islets (3%, 6%, 9%, 12%, and 17% of control, respectively). Similarly, the levels of Tcf2 (HNF-1β), glucokinase, Foxa1 (Forkhead box A1, HNF-3α), and Tcf4 transcripts were profoundly decreased in LRP5−/− islets (33%, 49%, 51%, and 59% of control, respectively). In contrast, the levels of glucose transporter 2 were unchanged and the insulin transcripts were increased by 30% in LRP5−/− islets.

Effects of Wnt on Glucose-Induced Insulin Secretion. LRP5 has been shown to bind Wnt and believed to act as a coreceptor for the Wnt signaling pathway. To determine the involvement of Wnt proteins in glucose-induced insulin secretion, we pretreated LRP5+/+ islets with CM from Wnt-3a-, Wnt-5a-, or parental vector-transfected L cells (neo-CM) (27). As shown in Fig. 5*A*, pretreatment of LRP5+/+ islets with Wnt-3a-, and Wnt-5a CM for 16 h markedly stimulated glucose-induced insulin secretion. This Wnt protein-stimulated glucose-induced insulin secretion was blocked by the addition of purified Frizzled-related protein-1 (*FRP1* gene product), a soluble antagonist for sFRP-1 (28).

In contrast, this stimulation of glucose-induced insulin secretion by Wnt-3a-CM was not seen in uninfected LRP5-deficient islets (data not shown) or in LRP5−/− islets infected with AdLacZ, whereas AdLRP5 infection restored the Wnt-3a-stimulated insulin secretion (Fig. 5*B*). These data demonstrate that Wnt-3a-stimulated glucose-induced insulin secretion is mediated by LRP5. In contrast to the stimulation by Wnt-3a of glucose-induced insulin secretion, the intracellular insulin levels

Table 2. Relative amounts of mRNAs in islets from LRP5−/− mice as compared with values in +/+ mice

mRNA from islets	Relative amount of mRNA in LRP5−/− mice
Tcf1 (HNF-1α)	0.17
Tcf2 (HNF-1β)	0.33
Tcf4	0.59
Foxa1 (HNF-3α)	0.51
Foxa2 (HNF-3β)	0.85
HNF-4α	0.09
Insulin	1.30
IRS-1	0.60
IRS-2	0.06
Insulin receptor	0.12
IGF-1	1.78
IGF-2	0.64
IGF-1 receptor	0.03
Glucose transporter 2	0.94
Glucokinase	0.49
LRP5	0.01

Male mice aged 6–8 months were used in this experiment. Total RNA from islets of four mice was pooled and subjected to real-time PCR quantification as described under *Experimental Procedures*. Cyclophilin was used as the invariant control. Values represent the amount of mRNA relative to that in LRP5+/+ mice, which is arbitrarily defined as 1.

were unchanged, indicating that Wnt-3a has no effects on the production of insulin in the islets (data not shown).

Discussion

Here, we investigated the function of LRP5 by examining LRP5−/− mice. We show that LRP5 is required for proper hepatic clearance of chylomicron remnants and for glucose-induced insulin secretion from the pancreatic islets, in addition to bone and eye development. Hyperlipoproteinemia has long been known to be a significant complication of diabetes, and our studies suggest a possible molecular linkage through LRP5. So far, the LRP5 locus has been linked to type 1 diabetes (32, 33) in humans, and our studies indicate that a more detailed investigation of the linkage of LRP5 with type 2 diabetes is warranted.

Consistent with the impaired glucose-induced insulin secretion and the reduced ATP/ADP ratio in LRP5−/− islets, the

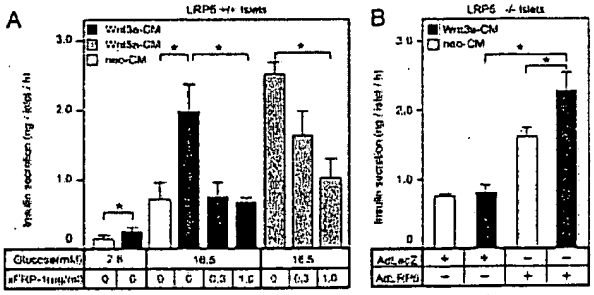

Fig. 5. Effects of Wnt-3a and Wnt-5a on glucose-induced insulin secretion. (*A*) Insulin secretion from the islets. LRP5+/+ islets were pretreated with 5-fold diluted Wnt-3a-CM, Wnt-5a-CM, or control neo-CM in the presence of the indicated concentration of sFRP-1 for 16 h before measuring insulin secretion induced by glucose. (*B*) Lack of Wnt-3a stimulation of insulin secretion from LRP5-deficient islets and restoration by AdLRP5. LRP5−/− islets were infected with AdLRP5 or AdLacZ and exposed to Wnt-3a- or control neo-CM for 16 h before measuring insulin secretion in the presence of 16.5 mM glucose. The values are the mean ± SE for four mice from quadruplicate determinations. *, $P < 0.01$; Student's *t* test.

steady-state levels of mRNAs for several important molecules in the islets were profoundly decreased. These include the HNF family of transcriptional factors (Tcf1, Tcf2, Foxa1, and HNF-4α,), glucose-sensing protein (glucokinase), and insulin-signaling proteins (insulin receptor, IGF-1 receptor, and IRS-2). Mutations in Tcf1, Tcf2, and HNF-4α genes impair insulin secretion and cause mature-onset diabetes of the young (MODY). Insulin signaling through the insulin receptor is important for maintaining the transcriptional level of glucokinase and insulin itself in beta cells (34–36). Also, the IGF-1 receptor signaling pathway through IRS-2 mediates the beta cell compensation for peripheral insulin resistance, as well as the development, proliferation, and survival of beta cells (37, 38). Our data provide the evidence that LRP5 together with Wnt maintains the normal function of the β cells through the transcriptional regulation of the above-mentioned genes.

Glycogen synthase kinase β (GSK3β) is a key component in many biological processes, including insulin- and Wnt-signaling pathways. Both insulin and Wnt inactivate GSK3β although through different mechanisms: phosphorylation mediated by Akt/PKB and axin conduction complex, respectively. Whereas insulin induces glycogen synthase activity through the inactivation of GSK3β, Wnt had no effect on glycogen synthase activity (39). Despite the marked reduction of steady-state insulin receptor transcripts in LRP5$-/-$ islets, the glycogen content of the islets, hepatic glycogen synthase activity, and pancreatic insulin content were almost unchanged in LRP5$-/-$ mice. In the beta cell-specific knockout for the insulin receptor, there is a decrease in glucose-stimulated insulin release and a marked reduction the insulin content of the cells (35). In contrast, the insulin content in the islets is unaltered in the beta cell-specific knockout for IGF receptor, whereas glucose-stimulated insulin secretion is markedly impaired (38). Based on the similarity of glucose-sensing defects between the mice lacking LRP5 and beta cell-specific IGF receptor, and the drastic reduction of IGF receptor transcripts in LRP5$-/-$ islets, it is suggested that IGF signaling is impaired in LRP5$-/-$ islets.

Despite the differences in the biological roles of Wnt-3a and Wnt-5a (β-catenin/Wnt pathway and Ca^{2+}/Wnt pathway, respectively), both proteins have similar effects on the stimulation of glucose-induced insulin secretion. Based on the stimulation of glucose-induced insulin secretion by Wnt proteins and the lack of the Wnt-stimulated insulin secretion in LRP5 deficient islets, we conclude that LRP5 together with Wnt proteins modulates glucose-induced insulin secretion. Although the precise pathway for Wnt signaling in the islets is currently unknown, this work has demonstrated that Wnt proteins are involved in normal glucose metabolism in the adult mouse and suggests that the Wnt pathway may provide novel therapeutic strategies for the treatment of type 2 diabetes.

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Severe Hypercholesterolemia, Impaired Fat Tolerance and Advanced Atherosclerosis in Mice Lacking Both LDL Receptor-Related Protein 5 (LRP5) and Apolipoprotein E *

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Running title: Mice lacking both LRP5 and apoE

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SUMMARY

LDL receptor-related protein 5 (LRP5) plays multiple roles including embryonic development and bone accrual development. Recently we demonstrated that LRP5 is also required for normal cholesterol metabolism and glucose-induced insulin secretion (Fujino *et al.,* Proc. Natl. Acad. Sci. USA vol. 100, 229-234, 2003). To further define the role of LRP5 in the lipoprotein metabolism, we compared plasma lipoproteins in mice lacking LRP5, apolipoprotein E (apoE), or both (apoE;LRP5 double knockout). On a normal chow diet, the apoE;LRP5 double knockout mice (older than four months of age) had approximately 60% higher plasma cholesterol levels compared with the age-matched apoE knockout mice. In contrast, LRP5 deficiency alone had no significant effects on the plasma cholesterol levels. HPLC analysis of plasma lipoproteins revealed that cholesterol levels in the VLDL and LDL fractions were markedly increased in the apoE;LRP5 double knockout mice. There were no apparent differences in the pattern of apoproteins between the apoE knockout mice and the apoE;LRP5 double knockout mice. The plasma clearance of intragastrically loaded triglyceride was markedly impaired by LRP5 deficiency. The atherosclerotic lesions of the apoE;LRP5 double knockout mice aged six months were approximately three-fold greater than those in the age-matched apoE-knockout mice. Furthermore, histological examination revealed highly advanced arthrosclerosis, with remarkable accumulation of foam cells and destruction of the internal elastic lamina in the apoE;LRP5 double knockout mice. These data suggest that LRP5

mediates both apoE-dependent and apoE-independent catabolism of plasma lipoproteins.

INTRODUCTION

Genetic defects in the catabolism of plasma lipoproteins are important causes of hypercholesterolemia and atherosclerosis in humans. The prototypic diseases are familial hypercholesterolemia, caused by a defect in the LDL receptor (LDLR) [1] (1), and familial type III hyperlipoproteinemia, caused by a defect in one of the ligands for LDLR, apolipoprotein E (apoE) (2).

ApoE is hypothesized to mediate lipoprotein clearance by binding two receptors: (*i*) LDLR and (*ii*) a hepatic chylomicron remnant receptor. ApoE deficient mice (3-5) and LDLR deficient mice (6) exhibit hypercholesterolemia, but the severity and manifestations differ markedly. On a normal laboratory chow diet, the apoE knockout mice have much more profound hypercholesterolemia and develop spontaneous atherosclerosis (4).

LDL receptor-related protein 5 (LRP5) is a member of the LDL receptor family that are characterized by the presence of cysteine-rich complement type ligand binding domains. LRP5 binds apoE-containing lipoproteins *in vitro,* and is widely expressed in many tissues including hepatocytes, adrenal gland and pancreas (7).

LRP5 and its homologue, LRP6, are postulated to play as co-receptors for Wnt receptors, Frizzled (8-13). The Wnt signaling pathway plays an essential role in embryonic development (14,15) and oncogenesis (16) through various signaling molecules including Frizzled receptors (17), LRP5 and LRP6 (8-13) and Dickkopf proteins (11,12,18). The Wnt signaling is also involved in adipogenesis by

negatively regulating adipogenic transcription factors (19). Recent studies have revealed that loss of function mutations in the LRP5 gene cause the autosomal recessive disorder osteoporosis-pseudoglioma syndrome (OPPG) (20). Consisting with human OPPG, LRP5 knockout mice generated by Kato *et al.* exhibit a severe low bone mass phenotype (21).

Recently, we demonstrated that LRP5 deficient mice develop high plasma cholesterol levels after feeding a high-fat diet, due to decreased hepatic clearance of chylomicron remnants (22). The hepatic clearance of apoE-rich chylomicron remnants was also markedly decreased in the LRP5 knockout mice. These data suggested that LRP5 plays a role in the hepatic clearance of chylomicron remnants. In addition, we showed that the LRP5 deficient mice fed a normal diet showed marked impaired glucose tolerance. The LRP5 deficient islets had a marked reduction in the levels of intracellular ATP and Ca^{2+} in response to glucose, thereby glucose-induced insulin secretion was decreased (22). Together with the roles of LRP5 in the bone accrual development (20,23,24) as well as in the Wnt signaling pathways (8-11,13), our data indicated that LRP5 is a multifunctional receptor physiologically linked to common human disorders, including hypercholesterolemia and impaired glucose tolerance.

To further define the role of LRP5 in lipoprotein metabolism, we produced double-knockout mice that are deficient in apoE as well as in LRP5 (apoE;LRP5 double knockout mice). In the current paper, we describe that superimposition of an LRP5 deficiency onto apoE deficiency increased plasma cholesterol beyond the level observed with apoE deficiency alone. We also show that fat tolerance was markedly

impaired in the LRP5 knockout mice as well as in the apoE;LRP5 double knockout mice. Consistent with extreme hypercholesterolemia, severe atherosclerosis developed in the apoE;LRP5 double knockout mice. These results provide further evidence for the role of LRP5 in the catabolism of plasma lipoproteins.

EXPEIMENTAL PROCEDURES

Materials—For the lipoprotein analysis, blood was collected from the retroorbital plexus after a four hours fasting. Plasma total cholesterol levels were determined in individual mice at each time point by enzymatic assay kits (Wako Pure Chemical Co, Japan).

For the detection of cholesterol and triglycerides with the HPLC method (see below), we obtained enzymatic reagents from Kyowa Medex Co. (Tokyo, Japan). The reagent system for cholesterol detection consists of reagent 1 (R1-C) and reagent 2 (R2-C): R1-C, 20 mM MOPS, pH 7.0, 1.1 mM *N*-ethyl-*N*-(3-methylphenyl)- *N'*-succinylethylendiamine (EMSE), 10 U/ml peroxidase, detergents, and stabilizer; R2-C, 20 mM MOPS, pH 7.0, 1.5 mM 4-aminoantipyrine, 0.68 mM $CaCl_2$, 0.3 U/ml cholesterol esterase, 2 U/ml cholesterol oxidase, 10 U/ml peroxidase, detergents, and stabilizer. The triglyceride reagent system includes reagent 1 (R1-TG) and reagent 2 (R2-TG): R1-TG, 50 mM PIPES, pH 6.2, 1.1 mM EMSE, 2 mM $MgSO_4$, 4.9 mM ATP, 3 U/ml glycerol kinase, 1.5 U/ml glycerol-3-phosphate oxidase 5 U/ml peroxidase, detergents and stabilizer; R2-TG, 50 mM PIPES, pH 6.2, 1.5 mM 4-aminoantipyrine, 2 mM $MgSO_4$, 3U/ml lipoprotein lipase, 5U/ml peroxidase, detergents, and stabilizer. Equal amounts of R1 and

R2 were mixed before use. After mixing, the cholesterol reagent was used within four weeks and the triglyceride reagent within two weeks.

Lipoprotein analysis by a dual detection HPLC system---Plasma lipoproteins were analyzed by an improved high performance liquid chromatography (HPLC) analysis according to the procedure as described by Usui *et al.* (25). The HPLC system consisted of an AS-8020 auto-injector, CCPS and CCPM-II pumps, and two UV-8020 detectors (Tosoh, Japan) (26). An SC-8020 system controller (Tosoh) was used for instrument regulation and data collection. Lipoproteins were fractionated on two tandem connected TSKgel LipopropakXL columns (300 x 7.8 mm, Tosoh) with 50 mM Tris-acetate, pH 8.0 containing 0.3 M sodium acetate, 0.05% sodium azide, and 0.005% Brij-35 at a flow rate of 0.7 ml/min. The TSK column medium is composed of porous polymermatrices with a nominal bead size of 10 μm and a pore size of 100 nm, which is expected to exclude most of chylomicron (CM) to the void volume. Two TSK columns were connected in tandem and used to obtain higher resolution within a relatively short analytical time. The running buffer was filtered through a 0.22 μm filter (Millipore Co., Bedford, MA) before use and continuously degassed with a SD-8022 on-line degasser (Tosoh) during analysis. The column effluent was split equally into two lines by a Micro-Splitter P-460 (Upchurch Scientific Inc., Oak Harbor, WA), one mixing with cholesterol reagent and the other with triglyceride reagent, in order to achieve simultaneous profiles from a single injection. The two enzymatic reagents were each pumped at a flow rate of 0.35 ml/min for the TSK column. Both enzymatic reactions proceeded at 37 °C in a reactor

coil (Teflon, 15 m x 0.4 mm id). Ten µl samples diluted with saline were injected by an AS-8020 auto-injector with a pre-suction volume of 25 µl at intervals of 24 min. The enzymatic determination of cholesterol and triglycerides involved the detection of hydrogen peroxide produced by cholesterol oxidase and glycerol-3-phosphate oxidase, respectively. Total cholesterol and triglyceride concentrations (in mg/dl) were calculated by comparison with total area under the chromatographic curves of a calibration material of known concentration.

SDS polyacrylamide gel electrophoresis---Total lipoprotein fractions ($d < 1.215$ g/ml) from pooled plasma of the mice were isolated by ultracentrifugation, and the delipidated apolipoproteins were boiled for 3 min in SDS sample buffer containing 2-mercaptoethanol, and subjected to electrophoresis on an SDS/5-15% polyacrylamide gel. Proteins were stained with Coomassie blue.

Fat Tolerance Test---Six-month-old male mice were fasted for 16 h and olive oil (1 ml/30 g body weight, Wako Pure chemicals Co., Osaka, Japan) was administered intragastrically as a bolus. Approximately 50 µl of blood was taken from the tail vein at the indicated times for the measurement of triglyceride levels and HPLC analysis.

Mice---LRP5 "knockout" mice (originally C57BL/6J-CBA hybrids, ref. 22), LRP5-/-, have been continually mated with C57BL/6J: N6 and N7 generation descendents from this cross into the C57BL/6J background were used. ApoE-/- mice (3) backcrossed ten times on the C57BL/6J background were obtained from the Jackson Laboratory (Bar Harbor, ME). To obtain knockout mice that are homozygous

for disruption of both the LRP5 and apoE loci, male apoE-/- mice were mated to female LRP5-/- mice. The resulting apoE+/-; LRP5+/- mice were identified by PCR analysis and bred each other to produce apoE-/-; LRP5-/- mice. Experiments were performed with those mice or those with the same genotype from the next generation by breeding apoE-/-; LRP5-/- each other. Mice were maintained on 12-h dark/12-h light cycles and had free access to a normal laboratory chow diet (4.5% fat, 0% cholesterol, CE-2, CLEA, Tokyo, Japan) and water.

Measurement of atherosclerotic lesions---Mice were euthanized, and thoracic and abdominal aorta were used for *en face* staining with oil Red O to visualize neutral lipid (cholesteryl ester and triglycerides) accumulation. In brief, the aorta was removed, cleaned and cut open with the luminal surface facing up, then immersion-fixed in 10% formalin in 10 mM PBS. After rinsing with PBS, the aorta was thoroughly cleaned of adventitial fat using micro forceps and spring iris scissors under a stereoscopic microscope. The inner aortic surface was stained with oil Red O for 25 min at room temperature. After rinsing with 60% isopropanol and distilled water, the oil Red O-stained area was quantified by NIH-Image 1.62 f software analysis of the digitized microscopic images. Results are expressed as percentage of lipid accumulating lesion area of the total aortic area analyzed.

For light microscopy, the aortic tissue samples were fixed with 10% formalin in 10 mM phosphate buffer (pH7.2) and embedded in paraffin. Sections 2-3-μm thick were taken longitudinally through the aortic lumen and stained with hematoxylin and eosin (HE) or elastica-Masson (EM). For oil Red O

staining, aortic tissue samples were frozen in OCT compound (Miles Inc., Elkhart, IN). Cryostat tissue sections were cut to a thickness of 5 μm and stained with oil Red O. Nuclei were counterstained with hematoxylin.

RESULTS

Plasma cholesterol and lipoprotein profile—Fig. 1 compares the levels of total cholesterol of mice of four different genotypes at the indicated ages. Mice were fed a normal laboratory chow diet containing 4.5% (w/w) fat and 0% cholesterol. Although there was no significant differences in the total plasma cholesterol levels between the apoE knockout mice (apoE-/-;LRP5+/+) and the apoE;LRP5 double knockout mice (apoE-/-;LRP5-/-) at two months of age, the cholesterol levels of the double knockout mice older than four months were greatly increased (approximately by 60%) beyond the levels observed with apoE deficiency alone. In contrast, LRP5 deficiency alone had no significant effects on the plasma cholesterol levels.

High resolution HPLC analysis (25) of plasma lipoprotein of four-month-old mice revealed that cholesterol levels in the VLDL and LDL fractions were markedly increased in the apoE;LRP5 double knockout mice compared with the apoE knockout mice (Fig. 1B and Table I): the cholesterol levels in the VLDL and LDL fractions in the apoE knockout were 180 ± 35 and 145 ± 7 mg/dl, respectively; and those in the apoE;LRP5 double knockout mice were 244 ± 24 and 171 ± 21 mg/dl, respectably (TABLE

I). There were no significant differences in the levels of chylomicron (CM)- and HDL-cholesterol between the apoE knockout mice and the apoE;LRP5 double knockout mice, although HLD-cholesterol levels in these mice were approximately 50% of those in the LRP5 knockout mice and normal controls. Despite the severe hypercholesterolemia in the apoE knockout and apoE;LRP5 double knockout mice, there were no significant differences in the total triglyceride levels among mice with the four different genotypes (data not shown).

Fig. 2 shows the SDS polyacrylamide gel electrophoresis of apoproteins in pooled lipoprotein fraction from mice of four different genotypes. Consistent with the previous work by Ishibashi *et al.* (27), the amounts of apoB48 were markedly increased in the apoE knockout mice as well as in the apoE;LRP5 double knockout mice. Despite the severe hypercholesterolemia in the apoE;LRP5 double-knockout mice, there were no apparent differences in the pattern of apoproteins between the apoE- and apoE;LRP5 double-knockout mice.

Fat tolerance test---In the pervious study, we have shown that LRP5 plays a role in the hepatic uptake of dietary cholesterol. The LRP5 knockout mice displayed dietary derived hypercholesterolemia due to decreased plasma clearance of chylomicron remnants (22). To further define the role of LRP5, fat tolerance test was carried out using mice of four different genotypes. Mice were fasted for 16 h and olive oil (1 ml/30 g body weight) was administered intragastrically. As shown in Fig. 3, plasma levels of total triglyceride increased and peaked at about 2 h, and then declined toward baseline 6 h after loading in both

apoE-knockout mice and normal controls. In contrast, the increased levels of plasma triglyceride were sustained for several h after loading in both LRP5 knockout and apoE;LRP5 double knockout mice, indicating that the plasma clearance of intragastrically loaded triglyceride was markedly impaired by LRP5 deficiency. HPLC analysis of plasma lipoproteins revealed that the majority of particles at 6 h after fat loading were in the VLDL fraction.

In addition, we noticed that 16 h fasting increased the levels of VLDL-triglyceride in the apoE knockout, LRP5 knockout and apoE;LRP5 double knockout mice. This result may indicate that both apoE and LRP5 mediate the plasma clearance of VLDL-triglyceride induced by fasting.

Atherosclerosis---Aortic atherosclerotic lesions of the apoE knockout and apoE;LRP5 double knockout mice were first analyzed by *en face* lipid staining (Fig.4A). At four months of age, the area of the thoracic and abdominal aortas stained by oil Red O of the apoE;LRP5 double knockout mice was approximately the same as that in the apoE knockout mice. In contrast, at six months of age, the lesions in the apoE;LRP5 double knockout mice were approximately three-fold larger than those in the apoE knockout mice (Fig.4B).

In histopathology under light microscopic examination, the lesions in the apoE knockout mice at six months of age were relatively modest, showing slightly atheromatous lesions with a fatty streak-like structure, which were localized on the surface of aortic intima, but were not associated with the destruction of internal elastic lamina or the medial muscle layer (Fig. 4C). In contrast, the apoE;LRP5

double-knockout mice developed multiple atheromatous lesions manifesting a hump structure, which were associated with cholesterin deposits, fibrosis and elastosis (Fig. 4D). Some of them showed the destruction of internal elastic lamina and the degenerative change of medial muscle layers of the aorta (Fig. 4E). In these lesions severe deposition of neutral lipid was observed (Fig, 4F).

DISCUSSION

In the present study, we show extreme hypercholesterolemia in mice lacking both apoE and LRP5. It has been well established that both LDLR and apoE are critical in the plasma clearance of cholesterol-carrying lipoproteins, including LDL and apoE-containing IDL and chylomicron remnants (1,2). In contrast to the mice lacking apoE (3-5) or LDLR (6), the lack of LRP5 alone did not increase the plasma levels of cholesterol on a normal diet, while high-fat feeding results in hypercholesterolemia in the LRP5 knockout mice (22). Ishibashi *et al.* showed that the plasma cholesterol levels in the double-knockout mice lacking both apoE and LDLR, were not significantly different from the levels in the apoE knockout mice (27). The severe hypercholesterolemia developed in the double knockout mice lacking both apoE and LRP5 suggests the presence of an alternative pathway for cholesterol catabolism mediated by LRP5, which appears to be independent of the LDLR pathway.

Consistent with the previous work (22), the LRP5 knockout mice and the apoE;LRP5 double knockout mice displayed markedly impaired fat tolerance. In contrast, the plasma clearance of intragastrically

loaded triglyceride was not significantly impaired in the apoE-knockout mice. These observations suggest that LRP5 modulates the plasma clearance of dietary derived triglycerides in the absence of apoE by stimulating the hydrolysis of triglycerides. In this context, it is important to refer that LRP5 and LRP6 can bind Dickkopf (Dkk), an antagonist of Wnt proteins (12, 24). Dkk is involved in *Xenopus* head formation and the impaired action of Dkk at LRP5 increases bone density in humans (24). The Dkk sequence consists of two cysteine-rich domains. The C-terminal domain has the typical cysteine pattern of colipase, which is required by pancreatic lipase for the efficient lipid hydrolysis (reviewed in ref. 28). The C-terminal domain of colipase binds to the C-terminal noncatalytic domain of pancreatic lipase, which is though to stabilize an active conformation of the lipase, and is also conserved among various lipases including, hepatic and lipoprotein lipases. Detailed sequence analysis and molecular modeling of the Dkk sequence onto the colipase structure suggest that Dkk and colipase have the same disulfide pattern and very similar three-dimensional structures (28). This structural analogy implies a common function (lipid interaction), and raises the possibility that Dkk bound to LRP5 stimulates lipid hydrolysis by interacting hepatic lipase and/or lipoprotein lipase. Furthermore, the impaired fat tolerance caused by the deficiency of LRP5 may lead to severe hypercholesterolemia in the absence of apoE.

Another explanation for the impaired lipoprotein metabolism in the apoE;LRP5 double knockout mice is that LRP5 may recognize other lipoproteins, in addition to apoE-containing lipoproteins. The candidate apoproteins that may be recognized by LRP5 remains unidentified, since the LRP5 deficiency

did not significantly alter the pattern of apoproteins in the plasma lipoproteins of the apoE knockout mice or that of normal mice.

In addition to the role of LRP5 in embryonic development and bone development, our current data provide further evidence that LRP5 also plays a role in the metabolism of plasma lipoproteins. Furthermore, consistent with the marked elevation of plasma cholesterol, severe arthrosclerosis developed in the apoE;LRP5 double-knockout mice. The remarkable destruction of the internal elastic lamina seen in the lesion of the double-knockout mice is characteristic of highly advanced atherosclerosis. The apoE;LRP5 double-knockout mice manifesting extreme hypercholesterolemia and highly advanced arthrosclerosis will provide a useful animal model for the research and development of therapeutic agents against hypercholesterolemia and atherosclerosis.

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FOOTNOTES

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[1] The abbreviations used are: apoE, apolipoprotein E; BSA, bovine serum albumin; CM, chylomicron; Dkk, Dickkopf; HDL, high density lipoprotein; HPLC, high performance liquid chromatography ; LDL, low density lipoprotein; LDLR,LDL receptor; LRP, LDL receptor-related protein; OPPG, osteoporosis-pseudoglioma syndrome; VLDL, very low density lipoprotein, PBS, phosphate-buffered saline.

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FIGURE LEGENDS

FIG. 1. **Age-dependent changes in plasma cholesterol concentrations in mice with different genotypes fed a normal diet.** *A*, Plasma levels of total cholesterol of mice of each genotype at the indicated age were determined enzymatically after 4 h fasting. Data are mean ± S.D. of six mice. * $P <$ 0.01; Student's *t* test. *B*, HPLC analysis of plasma lipoproteins. Plasma samples from mice of each genotype at four months of age were separated by HPLC, and cholesterol (solid line) and triglyceride (dashed line) contents were determined as described under "EXPERIMETAL PROCEDURES". Representative data from six animals with the indicated genotype is shown. The CM, VLDL, LDL, and HDL fractions are labeled C, V, L, and H, respectively. Free glycerol is indicated by an arrow head. The cholesterol levels in the CM, VLDL, LDL, and HDL fractions are shown in Table I.

Fig. 2. **SDS polyacrylamide gel electrophoresis of total lipoprotein fractions.** Equal volumes (1 ml) of plasma were pooled from four mice of different genotypes fed a normal diet and total lipoprotein fractions ($d < 1.215$ g/ml) were isolated by ultracentrifugation, and the delipidated apoproteins were subjected to electrophoresis on an SDS/5-15% polyacrylamide gradient gel. Proteins were stained with Coomassie Blue. Position of migration of apoB100, apoB48, apoA-VI, apoE, and apoA1 are denoted. Representative data from four independent experiments is shown.

Fig. 3. **Effects of intragastrical fat loading on plasma triglyceride levels in mice with different genotypes.** Six males (six-month-old) of each genotype received an intragastrically administration of olive oil (1 ml/30 g body weight). At the indicated times, 50 μl of blood was taken from the tail vein and subjected to HPLC analysis. Data are mean ± S.E. of six mice. * $P < 0.01$; Student's *t* test.

FIG. 4. **Atherosclerotic lesions in apoE- and apoE;LRP5 double-knockout mice.** *Panel A*, *En face* lipid staining of aortas. Thoracic and abdominal aorta from the indicated genotype was cut open with the luminal surface facing up, and the inner aortic surface was stained with oil Red O. Representative data of each genotype is shown. Bar = 5 mm. *Panel B*, Quantitative analysis of *en face* lipid staining. The inner aortic surface area stained with oil Red O was quantified by NIH-Image 1.62 f software analysis of the digitized microscopic images. Results are expressed as percentage of lipid accumulating lesion area of the total aortic area analyzed. Data are mean ± S.D. of six mice. * $P < 0.01$; Student's *t* test. *Panels C-F*, Representative histopathological features of the aorta. Bars: 100 μm. *C*, An apoE-knockout mouse (aged six months) shows a slightly atheromatous lesion characteristic of the accumulation of foam cells, which is not associated with the destruction of internal elastic lamina (dark brown-colored) or the degenerative change of muscle layer of the aorta (elastica-Masson staining). *D*, One of the multiple atheromatous lesions developed in an apoE;LRP5 double-knockout mouse (aged six months) manifests a hump structure associated with cholesterin deposits, fibrosis (light green-colored) and elastosis (dark brown-

colored). Destruction of the internal elastic lamina adjacent with a degenerative lesion of muscle layer of the aorta is remarkable (elastica-Masson staining). *E*, An atheromatous lesion in an apoE;LRP5 double-knockout mouse (aged six months) reveals a remarkable accumulation of foam cells, especially marked in the superficial region of atheroma, and a crystal structure of cholesterin deposits (hematoxylin and eosin staining). *F*, An atheromatous lesion in an apoE;LRP5 double knockout mouse (aged six months) reveals severe deposition of neutral lipid in the aortic wall resulting in the destruction of lamellar structure of the elastic fibers (oil red O staining).

TABLE I

Plasma cholesterol profiles in mice with different genotypes.

Plasma samples from mice of each genotype at four months of age were separated by HPLC, and cholesterol contents were determined as described under "EXPERIMENTAL PROCEDURES". Values are mean ± S.D. of six mice.

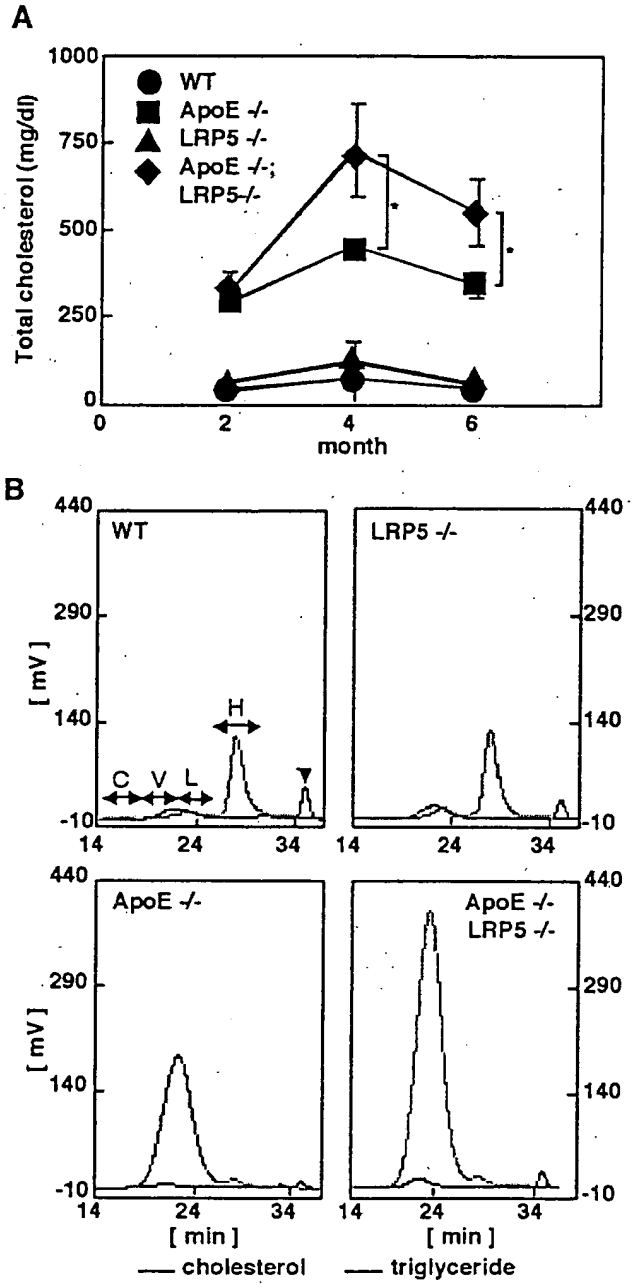
Genotype	CM	VLDL	LDL	HDL
		Cholesterol mg/dl		
ApoE+/+; LRP5+/+	0.10 ± 0.12	2.26 ± 0.28	4.59 ± 1.05	39.9 ± 2.8
ApoE+/+; LRP5-/-	0.03 ± 0.02	3.58 ± 0.40	6.11 ± 1.01	41.2 ± 1.5
ApoE;LRP5+/+	0.16 ± 0.08	180 ± 35 [a]	145 ± 7 [a]	21.7 ± 3.8 [c]
ApoE;LRP5-/-	0.12 ± 0.05	244 ± 24 [a, b]	171 ± 21 [a, b]	22.9 ± 1.6 [c]

[a] $P < 0.01$ vs. ApoE+/+; LRP5+/+ and ApoE+/+; LRP5-/-

[b] $P < 0.01$ vs. ApoE-/-;LRP5+/+

[c] $P < 0.01$ vs. ApoE+/+; LRP5+/+ and ApoE+/+; LRP5-/-

Fig.1


A
1000
750
500
250
0
Total cholesterol (mg/dl)
WT
ApoE -/-
LRP5 -/-
ApoE -/-;
LRP5-/-
0
2
4
6
month
B
440
290
140
-10
[mV]
WT
C V L
H
LRP5 -/-
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LRP5 -/-
14
24
34
[min]
cholesterol
triglyceride

Fig.2

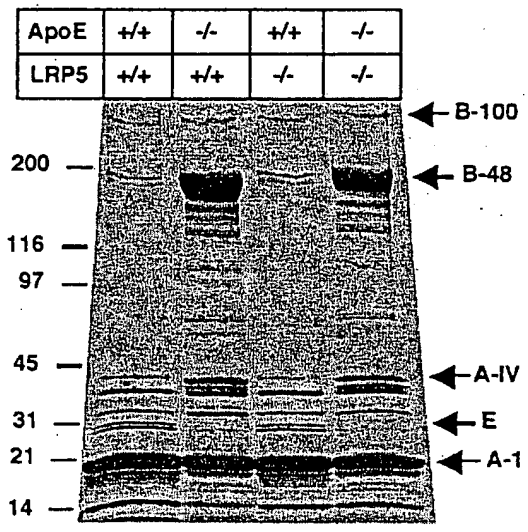


Fig.3

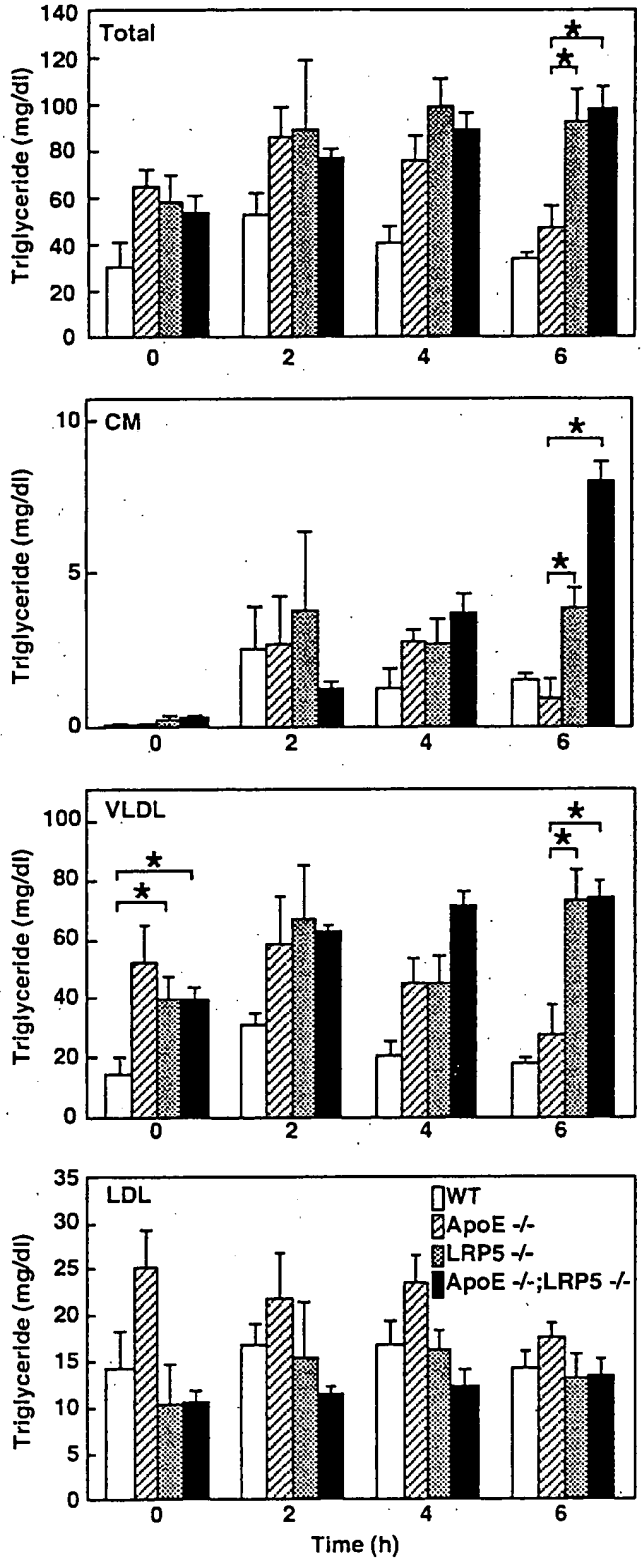
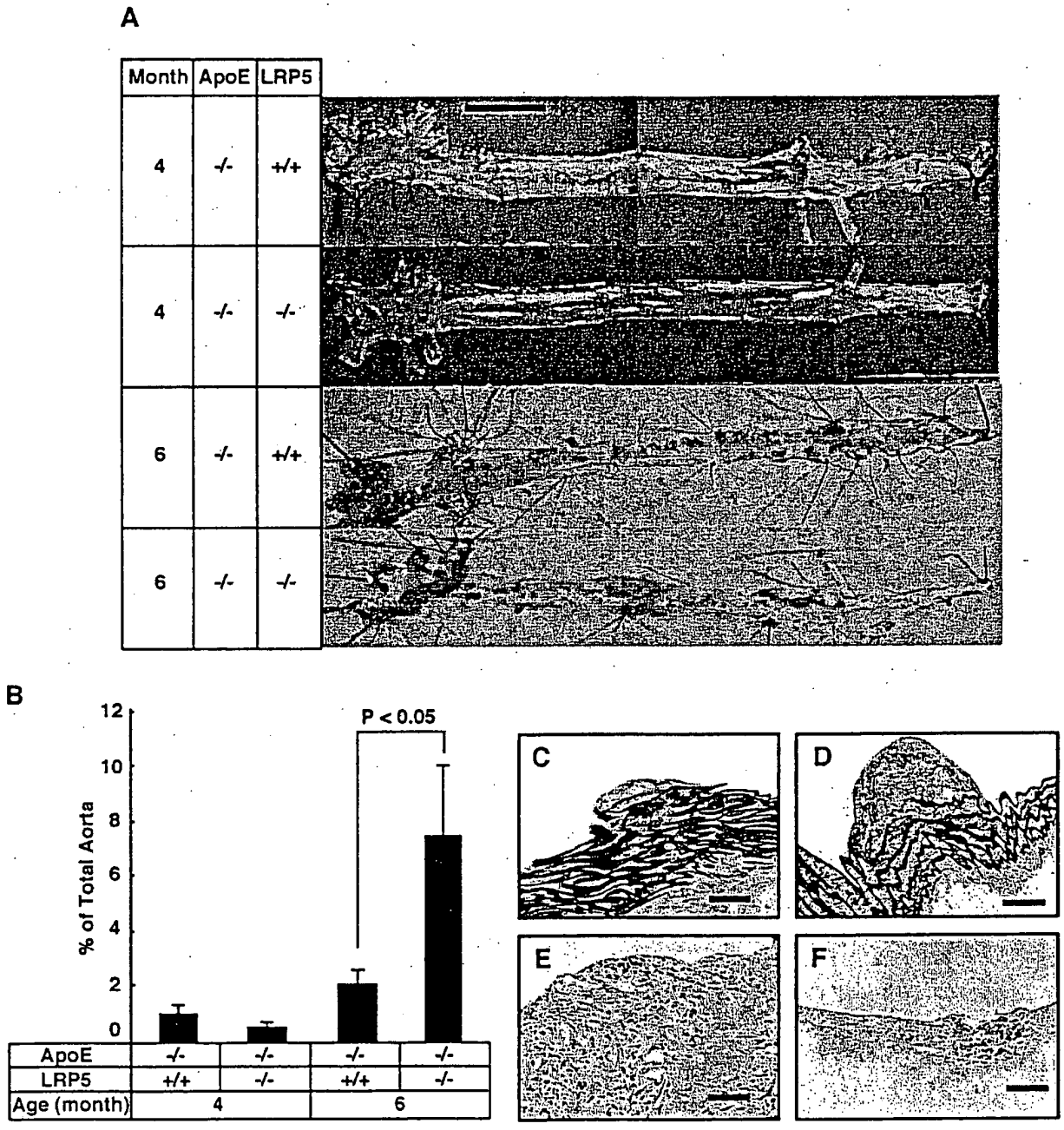

Total
CM
VLDL
LDL
Triglyceride (mg/dl)
Time (h)
WT
ApoE -/-
LRP5 -/-
ApoE -/-;LRP5 -/-
*

Fig.4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Lipases and HDL metabolism

Weijun Jin, Dawn Marchadier and Daniel J. Rader

Plasma levels of high-density lipoproteins (HDL) cholesterol are strongly inversely associated with atherosclerotic cardiovascular disease, and overexpression of HDL proteins, such as apolipoprotein A-I in animals, reduces progression and even induces regression of atherosclerosis. Therefore, HDL metabolism is recognized as a potential target for therapeutic intervention of atherosclerotic vascular diseases. The antiatherogenic properties of HDL include promotion of cellular cholesterol efflux and reverse cholesterol transport, as well as antioxidant, anti-inflammatory and anticoagulant properties. The molecular regulation of HDL metabolism is not fully understood, but it is influenced by several extracellular lipases. Here, we focus on new developments and insights into the role of secreted lipases on HDL metabolism and their relationship to atherosclerosis.

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HDL (see Glossary) play an important role in cholesterol homeostasis. They also protect the arterial wall from the development of atherosclerosis. One mechanism by which HDL protect is by promoting efflux of excess cholesterol from cells in the arterial wall, returning it to the liver for excretion into the bile, a process known as 'reverse cholesterol transport' [1,2]. However, there is evidence that HDL can protect LDL from oxidation [3], reduce the inflammatory response of endothelial cells [4,5], inhibit the coagulation pathway [6] and promote the availability of nitric oxide [7], so there might be other mechanisms by which HDL also protect against atherosclerosis.

HDL are macromolecules comprising lipids (phospholipids, cholesterol and some triglyceride), as well as apolipoproteins, the major one of which is apoA-I [8,9], which is synthesized and secreted by both the intestine and the liver. Nascent apoA-I-containing HDL particles interact with peripheral cells and acquire cholesterol and phospholipid through a transport process facilitated by the cellular protein ABCA1 (Fig. 1). Unesterified cholesterol is esterified to cholesteryl ester within the HDL particle by the enzyme LCAT. HDL cholesteryl ester can be taken up selectively by the liver through the action of the SR-BI. Cholesteryl ester can also be selectively transferred to apoB-containing lipoproteins in exchange for triglyceride through the action of CETP. Conversely, the PLTP mediates transfer of phospholipids from apoB-containing lipoproteins to HDL. Several members of the family of triglyceride lipase genes also influence the metabolism of HDL. Hydrolysis of triglycerides in triglyceride-rich lipoproteins by LPL results in transfer of lipids and apolipoproteins to HDL. HL hydrolyzes HDL triglyceride and phospholipids, generating smaller lipid-depleted HDL particles. Finally, EL might hydrolyze HDL phospholipids, thus promoting HDL catabolism. sPLA2 also has the ability to hydrolyze HDL phospholipids (Fig. 1). Here, we discuss the secreted lipases that are directly involved in HDL metabolism and detail the new developments in the field.

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Lipoprotein lipase

LPL is synthesized in adipocytes and in both skeletal and cardiac myocytes. It is transported to the capillary endothelial surface where, bound to HSPGs, it hydrolyzes triglycerides in the triglyceride-rich lipoproteins, chylomicrons and VLDL [10]. Through its lipolytic action, LPL generates FFAs for conversion to triglyceride to be stored in adipocytes, and for energy use by skeletal and cardiac myocytes, and thus plays an important role in energy homeostasis [11]. After lipolysis of triglyceride-rich lipoproteins, surface phospholipids and apolipoproteins from these lipoproteins dissociate and are acquired by HDL. Indeed, transgenic overexpression of the gene encoding LPL in mice (*Lpl*) results in increased HDL cholesterol (HDL-C) levels [12]. Conversely, the gene knockout of *Lpl* is associated not only with severe hypertriglyceridemia, but also with very low HDL-C levels in targeted mice [13,14]. In *Lpl*-deficient mice rescued by crossbreeding with mice expressing LPL in cardiac muscle alone, plasma triglycerides and HDL-C levels were normalized in adult animals [15]. However, although mice expressing LPL only in skeletal muscle have normal triglycerides, they have reduced HDL-C levels compared with wild-type mice [16]. Therefore, the tissue source of the LPL might be an important determinant of its effects on HDL.

In humans, deficiency of LPL is also associated with severe hypertriglyceridemia and very low HDL-C levels [17]. Even heterozygosity for LPL deficiency in humans is associated with reduced HDL-C levels [18]. Post-heparin plasma LPL activity is directly correlated with plasma HDL-C levels [19]. Interestingly, the relatively common LPL variant Ser447Stop is associated with increased LPL activity and increased HDL-C levels [18,20].

The relationship between LPL expression and atherosclerosis is complex, and could depend on the cellular origin of the LPL. Transgenic overexpression of *Lpl* in multiple tissues, including muscle and adipose, was associated with increased HDL-C levels and reduced atherosclerosis in

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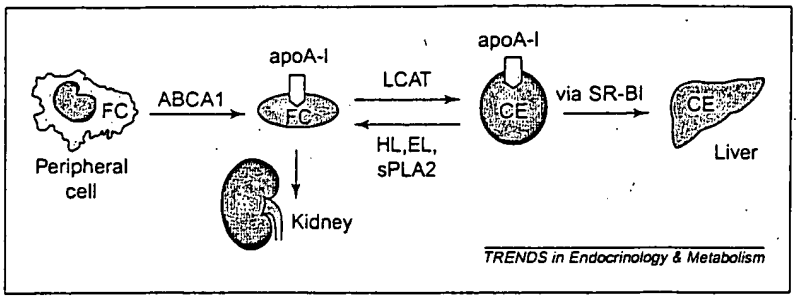

Fig. 1. A schematic diagram depicting the role of lipases in HDL metabolism. Lipid-poor apoA-I acquires free cholesterol (FC) from peripheral cells through an efflux process facilitated by the cellular protein ATP-binding cassette protein A1 (ABCA1). FC is converted to cholesteryl ester (CE) within the HDL particle through transfer of a fatty acid from phosphatidylcholine by the enzyme lecithin cholesterol acyltransferase (LCAT). HDL-CE can be taken up selectively by the liver through the action of the scavenger receptor class BI (SR-BI) and targeted for excretion in the bile. HDL-CE can also be selectively transferred to apoB-containing lipoproteins through the action of cholesteryl ester transfer protein and returned to the liver via the LDL receptor. Hepatic lipase (HL) hydrolyzes HDL triglyceride and phospholipid, remodeling larger HDL particles to smaller HDL particles, which are then at greater risk of catabolism via the kidney. Endothelial lipase (EL) and secretory phospholipase A2 (sPLA2) might also participate in the remodeling of HDL from larger to smaller particles. Lipoprotein lipase (LPL) is not shown but contributes to HDL formation by generating redundant phospholipids and apolipoproteins on apoB-containing lipoproteins that are transferred to HDL. Abbreviations: see Glossary.

Ldlr-deficient mice [21], *Apoe*-deficient mice [22] and cholesterol-fed rabbits [23]. Pharmacological upregulation of *Lpl* expression in rats was found to raise HDL-C levels and reduce atherosclerosis [24]. However, macrophage-derived *Lpl* was shown through bone marrow transplant studies to promote atherosclerosis without effects on HDL-C levels in mice [25], suggesting that the source of LPL expression could influence its effects on atherosclerosis. Overall, LPL derived from adipose tissue and muscle appears to increase HDL-C levels indirectly through its effects on triglyceride-rich lipoproteins and, when expressed in these tissues, it therefore appears to have antiatherogenic properties.

Hepatic lipase

HL is a member of the same triglyceride lipase gene family as LPL [26]. As its name suggests, HL is synthesized primarily in hepatocytes and is localized to the hepatic sinusoids, where it is bound to HSPGs [27]. HL hydrolyzes triglycerides and probably phospholipids in VLDL remnants or IDL, leading to more efficient uptake of these remnant particles and the generation of LDL. HL also acts on LDL to generate a small dense form of LDL. Finally, HL hydrolyzes triglycerides and probably phospholipids in the larger less-dense form of HDL, called HDL_2, thus promoting its remodeling to the smaller denser HDL_3 [28,29]. HL cooperates with other gene products, such as apoA-II, apoE, LCAT, CETP and SR-BI, in its influence on HDL metabolism. The effects of HL on HDL metabolism might be partially inhibited by apoA-II [30–32].

Data from animals and humans are consistent with the concept that HL influences HDL metabolism. Transgenic mice [33–35] and rabbits [36,37] that overexpress the gene encoding HL (*Lipc*) have decreased levels of HDL-C and smaller HDL particles (as well as reduced levels of apoB-containing lipoproteins). Overexpression of *Lipc* in the liver using adenoviral-mediated gene transfer reduced HDL-C levels by ~65% in *Lipc*-deficient mice [38], 63% in *Lcat*-transgenic mice [39] and 41% in *Apoe*-deficient mice [40]. Although the catalytic activity of HL plays a role in its effects on HDL after overexpression, expression of a catalytically inactive form of HL in mice also reduced HDL-C levels by 42% [41]. This was attributed to a bridging effect by which HL mediates the binding of HDL to the hepatocyte surface by bridging between HDL and the HSPGs on the cell surface. *In vitro*, expression of *Lipc* increased SR-BI-mediated HDL cholesterol ester uptake, an effect that was caused by both the lipolytic and the bridging functions of HL. *Lipc*-deficient female mice have levels of HDL-C elevated by ~10% [42].

In humans, high plasma HL activity is associated with reduced HDL-C levels and smaller HDL particles [19]. Conversely, genetic HL deficiency is associated with modestly elevated HDL-C levels and larger HDL particles [43,44]. A common single nucleotide polymorphism in the HL promoter has been associated with lower levels of HL activity and increased levels of HDL-C, especially HDL_2 [45–47], but this finding has not been replicated in all populations [48]. Nevertheless, genetic variation at *Lipc* is thought to be an important source of variation in HDL-C levels in the general population. The relationship between HL and atherosclerosis is complex. Overexpression of *Lipc* in mice has been reported to reduce atherosclerosis as assessed by aortic cholesterol content [33]. Conversely, knockout of *Lipc* expression in mice is associated with reduced atherosclerosis in *Apoe*-deficient mice [49,50]. HL-deficient humans have been reported to be at increased risk for atherosclerotic vascular disease [44], but also have elevated levels of atherogenic lipoproteins. Humans with coronary heart disease have been reported to have increased [51] or decreased [52] post-heparin plasma HL activity levels compared with control subjects in cross-sectional studies. The regression of coronary atherosclerosis resulting from intensive lipid-lowering therapy is associated with reduction in HL activity and favorable changes in LDL buoyancy [53]. Therefore, there might be an optimal level of HL activity with regard to atherosclerosis: whereas having too little HL could impair remnant clearance and increase cardiovascular risk, having too much reduces HDL-C levels and increases risk.

Endothelial lipase

EL is a member of the same family of triglyceride lipases as are LPL and HL. It was cloned in a

human monocyte cell line (THP-1) by differential display after oxidated-LDL treatment [54], and independently by subtractive hybridization from human umbilical vein endothelial cells undergoing tube formation to monolayer [55]. It shares 45% identity with LPL, 40% identity with HL and contains three conserved catalytic residues, ten conserved cysteine residues, a 19-residue lid, four clusters of heparin-binding regions and five potential *N*-linked glycosylation sites. EL has triglyceride lipase activity, but relative to LPL and HL has substantially greater phospholipase activity, placing it at the other end of the lipolytic spectrum from LPL [56]. Overexpression of the gene encoding human EL (*LIPG*) in the livers of mice with a recombinant adenoviral vector markedly reduced plasma concentrations of HDL-C and apoA-I [54]. Although more work is required, these results suggest that EL could play a role in HDL metabolism by hydrolyzing HDL phospholipids.

Secretory phospholipase A2 group IIA

The sPLA2 family is a group of low-molecular weight secreted phospholipases [57]. The group IIA member of this family is the best known and is frequently referred to as sPLA2-IIA or simply sPLA2. It is an acute-phase protein that exhibits phospholipase activity at the sn-2 position on the phospholipid. Plasma levels of sPLA2-IIA are increased dramatically in patients with acute inflammatory conditions, such as bacterial infections, sepsis and multiorgan failure, but are also elevated in patients with chronic inflammatory diseases [58,59]. The hydrolysis of acute-phase HDL was twice and three-times more rapid and intense than that of normal HDL [60]. Transgenic overexpression of the gene encoding human sPLA2-IIA in mice results in reduced HDL-C levels [61], reduced HDL size [62], altered HDL composition [62] and increased rate of catabolism of HDL apolipoproteins and HDL-cholesterol esters [62]. *In vitro* studies using Chinese hamster ovary cell lines transfected with the gene encoding SR-BI showed that sPLA2-IIA-modified HDL was nearly twice as efficient as a substrate for cholesteryl ester transfer [63]. Interestingly, HDL-C and apoA-I levels are decreased in both acute and chronic inflammatory states [9], but the mechanisms behind this observation are poorly understood. Upregulation of the expression of the gene encoding sPLA2-IIA in both acute and chronic inflammatory states (such as in atherosclerosis itself) could be one cause of the reduced HDL-C levels associated with inflammation.

Lecithin cholesterol acyltransferase

LCAT transfers a fatty acid from phospholipid to unesterified cholesterol, thus resulting in the generation of cholesteryl ester. Although not classically thought of as a lipase, LCAT acts as a phospholipase in that the first step of the LCAT reaction is hydrolysis of phospholipid to generate the fatty acid used for generating the cholesteryl ester [64]. LCAT is found primarily on HDL, and is responsible for generating HDL cholesteryl ester, thereby having a major influence on HDL metabolism [65]. Transgenic overexpression of *Lcat* in mice [66,67] and rabbits [68] results in substantial increases in HDL-C levels. Overexpression of human *Lcat* in human *apoA-I*-transgenic [66,67] and human *apoA-I/apoA-II*-double transgenic mice [67] leads to even greater increases in the plasma concentrations of a cholesteryl ester-enriched, large HDL. Overexpression of *Lcat* in liver with recombinant adenovirus also increases HDL-C levels in mice [69]. Transgenic overexpression of human or murine *Lcat* in wild-type cholesterol-fed mice resulted in increased apoE-containing, cholesteryl ester-enriched HDL_1 and reductions in the concentrations of pre-β HDL, [66]. The overexpression of *Lcat* delayed the catabolism of apoA-I [70] and HDL cholesteryl ester [71] in rabbits. Conversely, *Lcat*-deficient mice have markedly reduced levels of HDL-C and apoA-I [72,73].

LCAT deficiency in humans is also associated with markedly reduced HDL-C and apoA-I levels [74]. HDL metabolic studies in LCAT-deficient humans demonstrated dramatically increased catabolic rates of apoA-I and especially apoA-II [75]. The role that LCAT plays in influencing HDL-C levels in the general population is uncertain. Some studies in humans have shown a positive correlation between HDL-C levels and LCAT activity [76] or LCAT mass [77].

The relationship of LCAT to atherosclerosis is also complex. Overexpression of human *Lcat* in cholesterol-fed rabbits was associated with markedly reduced atherosclerosis [78], but this effect requires the presence of functional LDLR [79]. By contrast, transgenic overexpression of human *Lcat* in mice either resulted in increased atherosclerosis [80] or afforded no evidence of protection from it [81]. Rabbits, like humans, express a functional CETP, whereas mice lack functional CETP in plasma. When human *Lcat* was overexpressed in mice that were also transgenic for human *Cetp* expression, cholesterol-induced atherosclerosis was significantly reduced compared with non-*Lcat* transgenic mice [82], suggesting that the antiatherogenic effect of LCAT requires the presence of CETP. The impact of LCAT deficiency on atherosclerosis in mice is uncertain. In one report, aortic atherosclerosis was significantly reduced in three different mouse models with *Lcat* deficiency (*Ldlr*-deficient, *Apoe*-deficient and *Cetp*-transgenic) [73]. However, in another model, LCAT deficiency was associated with increased atherosclerosis in *Ldlr*-knockout and

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Glossary

ABCA1: ATP-binding cassette protein A1
apoA-I: apolipoprotein A-I
CETP: cholesteryl ester transfer protein
EL: endothelial lipase
FFA: free fatty acids
HDL: high-density lipoproteins
HL: hepatic lipase
HSPG: heparan sulfate proteoglycans
IDL: intermediate-density lipoproteins
LCAT: lecithin-cholesterol acyltransferase
LDL: low-density lipoproteins
LDLR: low-density lipoprotein receptor
LPL: lipoprotein lipase
PLTP: phospholipid transfer protein
sPLA2: secretory phospholipase A2
SR-BI: scavenger receptor BI
VLDL: very low-density lipoproteins

Apoe-knockout mice [83]. Interestingly, in humans, LCAT deficiency is not obviously associated with increased risk of atherosclerosis [74]. Therefore, although LCAT clearly has important effects on HDL metabolism, its relationship to atherosclerosis remains unclear.

Conclusion

HDL metabolism is complex and regulated by several factors. Remodeling of the HDL particle within the plasma compartment by secreted lipases is one crucial component of the metabolism of HDL. These lipases play a major role in determining the steady-state levels of HDL-C as well as influencing the function of HDL particles. Because of their intimate relationship with HDL metabolism and function, they are likely to have important effects on atherosclerosis in humans. Indeed, several of these lipases are viable targets for new drug development.

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